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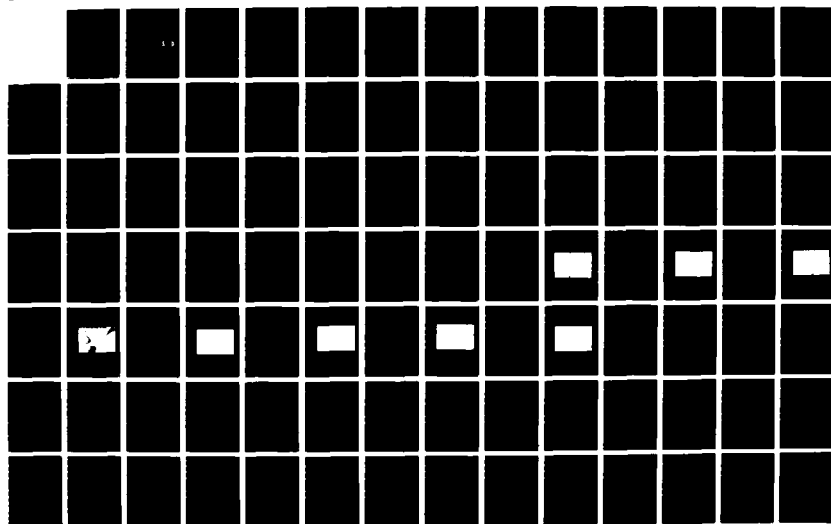
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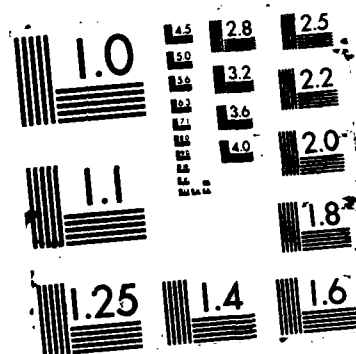
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MESENCHYMAL TISSUE RESPONSE TO HETEROTOPICALLY PLACED  
DEMINERALIZED BONE POWDER PARTICLES IN THE RAT

A  
THESIS

Presented to the Faculty of  
The University of Texas Graduate School of Biomedical Sciences  
at San Antonio  
in Partial Fulfillment  
of the Requirements  
for the Degree of  
MASTER OF SCIENCE

By  
Faye Marie Thery Fegley, M.S.

San Antonio, Texas

July 1987



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**MESENCHYMAL TISSUE RESPONSE TO HETEROTOPICALLY PLACED  
DEMINERALIZED BONE POWDER PARTICLES IN THE RAT**

**Faye Marie Thery Fegley**

**APPROVED:**

*Barbara D. Bryan*  
\_\_\_\_\_  
**Supervising Professor**

*Michael J. Kelly*  
\_\_\_\_\_

*James J. Lane*  
\_\_\_\_\_

*Robert J. Pele*  
\_\_\_\_\_

*David D. DePaola*  
\_\_\_\_\_

*7/13/87*  
\_\_\_\_\_  
**Date**

**APPROVED:**

*D. P. DePaola*  
\_\_\_\_\_  
**D. P. DePaola, D.D.S., Ph.D.**  
**Dean**

## DEDICATION

I dedicate this work to my best friend and husband Don, whose understanding, humor, encouragement, support, and love helped me through yet one more goal.

## ACKNOWLEDGEMENTS

I wish to thank my mentor and friend, Dr. Barbara Boyan, for her assistance and guidance which have made this endeavor possible. Additional thanks are given to the members of my committee, Drs. Robert Klebe, David Rohrbach, Michael Mills, and James Lane for their advice and contributions in the preparation of this thesis. Special acknowledgement and gratitude is extended to Drs. John Rapley and David Carnes for their professional advice and assistance and also for the moral support which led me to the completion of this thesis.

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MESENCHYMAL TISSUE RESPONSE TO HETEROTOPICALLY PLACED  
DEMINERALIZED BONE POWDER PARTICLES IN THE RAT

Faye Marie Thery Fegley, M.S.

The University of Texas Graduate School of Biomedical  
Sciences at San Antonio

Supervising Professor: Barbara D. Boyan, Ph.D.

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Urist (1965) has shown that demineralized bone fragments would stimulate osteogenesis even when implanted in heterotopic tissues. Demineralized bone powder (DBP) induces osteogenesis by promoting transformation of fibroblasts to chondroblasts with subsequent endochondral ossification (Reddi et al., 1973; Mulliken et al., 1980; Kaban et al., 1981). An important early event in heterotopic bone induction is the binding of fibronectin to DBP. Fibronectin is bound to DBP from 3-18 hours after implantation (Reddi, 1984). Since fibronectin is chemotactic for fibroblasts and acts as a substrate for

their migration (Kurkinen, 1980), it is possible that its presence at the time of DBP implantation might enhance the production of bone.

In a blind study, 188 Long Evans rats (2 months old) were divided into two groups in which each rat received four DBP implants placed in subcutaneous pouches of the thorax. One group received DBP particles that were incubated in phosphate buffered saline (PBS) containing rat serum fibronectin for 24 hours prior to implantation. The other group received DBP particles that were incubated in PBS only for 24 hours prior to implantation.

To assess the effect of recipient age on the tissue response to the DBP and to determine the effect of 'wetted' versus 'dry' implantation of the DBP particles, 4 three week old Long Evans rats each had four implants of DBP placed in subcutaneous thoracic pouches. Two of the four implants consisted of DBP 'wetted' with PBS 24 hours prior to implantation and two implants were placed as dry particles.

All of the implants which were implanted after incubation either in PBS alone or PBS with fibronectin, failed to induce bone formation regardless of the age of the recipient rat. Implants preincubated with fibronectin did exhibit a higher protein content initially. However, no differences in the rate or magnitude of response of mesenchymal tissues to either of the 'wetted' DBP preparations was observed. Unlike the response of mesenchymal tissue described by Reddi (Reddi et al., 1972),

only one peak of alkaline phosphatase, at Day 14, was observed coincident with the appearance of disorganized woven bone. No evidence of cartilage was detected at any time. The implants that were harvested at twenty-eight days after implantation were characterized histologically by a dense fibrillar network of collagen coursing between the DBP particles and this appearance is indicative of fibrosis. In contrast, DBP that was implanted as dry particles in the 3 week old rats formed cartilage and bone. These data indicate that exogenous serum fibronectin in PBS present at the time of implantation does not promote the formation of heterotopic bone and that prewetting with PBS alters the mesenchymal response to DBP. This may be due to a change in the required spacial relationship and proximity of the DBP particles or to the extraction of one or more inductive factors. *Hess* ←

### VITA

Faye Marie Thery Fegley was born on January 10, 1947 to Bernice Nyland Thery and Ignatius Herbert Thery, in Minneapolis, Minnesota. Graduating from St. Anthony of Padua High School, Minneapolis, Minnesota in June 1965 she enlisted in the United States Air Force as a dental assistant and military trained dental hygienist. On September 2, 1967 she was married to Donald J. Fegley of Shamokin, Pennsylvania. Upon completion of her enlisted service obligation, she attended the University of Minnesota from 1970-1972 and obtained her dental hygiene certificate. From 1972-1976 she was engaged in the practice of dental hygiene as well as attending Midwestern State University in Wichita Falls, Texas. In August of 1976, she was admitted to The University of Texas Health Science Center at San Antonio Dental School and received her Doctor of Dental Surgery degree in May of 1980. She entered the United States Air Force again in July 1980 and began a one year General Dentistry Residency at Langley AFB, Virginia. In August of 1981, she was assigned as a staff general dentist to RAF Upper Heyford, England. In June of 1984, she entered the Post-Doctoral Periodontics program at The University of Texas Health Science Center in San Antonio in conjunction with Wilford Hall USAF Medical Center. She was admitted to candidacy for the Master of Science degree at the Graduate School of Biomedical Sciences in April of 1985.

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## I. INTRODUCTION

When periodontal disease destroys alveolar bone, one additive treatment modality is the placement of bone grafts in the periodontal osseous defect in hopes of regenerating new bone and attachment. The use of demineralized bone in the treatment of osseous defects is not a new idea. As early as the 1800s, treatment of osteomyelitis was reported using xenogeneic demineralized bone (Senn, 1889). In 1965, Urist reported that demineralized bone fragments would stimulate osteoinduction in experimental sites, even when implanted in heterotopic tissues. Unlike lyophilized mineralized bone or bone marrow implants, demineralized bone powder induces osteogenesis by transformation of mesenchymal cells to chondroblasts with subsequent endochondral ossification (Reddi et al., 1973; Mulliken et al., 1980 and Kaban et al., 1981).

The osseous defects present in periodontal alveolar destruction present a unique and difficult environment for treatment by regenerative procedures. The oral environment is constantly bathed in saliva and indigenous bacteria which have the ability to interact with the healing tissue site and potentially alter wound healing. Also, epithelial cell proliferation may outdistance the connective tissue repair within the healing site preventing a functional regeneration of connective tissue components. In such an environment, enhancement of the rate of connective tissue differentiation

and osteogenesis may be beneficial. It is by augmenting the connective tissue response in the earliest stage of wound healing that one could hope to enhance the connective tissue response.

During the initial stage of healing, fibronectin (Kurkinen, 1980) acts as a substrate and chemoattractant for the migration of fibroblasts and endothelial cells into the wound site. Reddi (1984) has studied the biochemical activities that accompany the formation of endochondral bone formation and has found binding of fibronectin to DBP 3-18 hours after implantation of the matrix. Since it is the mesenchymal fibroblast that is transformed into a chondroblast by the inductive factors in demineralized bone powder, enhancement of fibronectin activity in effect may accelerate the fibroblastic response at the implantation site. For example, if fibronectin could be present immediately upon implantation of the demineralized bone powder (DBP), the fibronectin may accelerate the rate of migration of fibroblasts and ultimately enhance the formation of endochondral bone formation.

The question arises as to whether the addition of exogenous fibronectin, prior to the implantation of the demineralized bone matrix, could enhance the rate of the mesenchymal tissue differentiation into chondroblasts over that response normally generated by endogenous fibronectin present at the wound site. This point of inquiry was tested using an in vivo biologic system. Tissue healing response

was evaluated biochemically and histologically.

## II. LITERATURE REVIEW

### A. Fibronectin

Fibronectin is a cell surface and blood glycoprotein that appears to act as a connector. Fibronectin molecules can assemble into fibrils, bind to cells and link cells to other kinds of fibrils in the extracellular matrix. Fibronectin's adhesive character makes it a critical component in blood clots and as a substrate for migrating cells. In addition to capabilities as a connector, the chemoattractant abilities of fibronectin have been extensively studied (Pearlstein, 1976; Engvall and Ruoslahti, 1977; Seppa, et al., 1980; Gauss-Muller, et al., 1980).

Fibronectin is present in an insoluble form on cell surfaces and as a soluble form in plasma (300ug/ml). The soluble form of fibronectin was first described in the 1940s (Morrison, et al., 1948) and has since been described in a number of tissues and species. Due to the wide variety of places this protein has been located, it has been referred to by many names: LETS (large external transformation-sensitive protein (Hynes, 1973), cold-insoluble globulin, galactoprotein, as well as others. Chemically, the soluble forms appear to be identical to the fibronectin isolated from the fibroblast surface (Ruoslahti, et al., 1980). Differences observed in the electrophoretic motilities of fibronectins from different sources are mainly due to

variations in the glycosylation of the molecule and it is likely that the different forms of fibronectin, i.e., plasma and cellular, represent a single gene product present in different compartments of the body in slightly modified forms (Yamada and Olden, 1978).

Biochemically, the molecule of fibronectin is a dimer consisting of two similar subunits (Hynes and Destree, 1977; Keski-Oja, et al., 1977; Yamada, et al., 1978; and Engvall, et al., 1977). Each subunit has a molecular weight of 200,000 to 250,000 daltons which are joined at one end by disulfide bonds. The protein chain of each subunit forms an elongated structure 60-70 nanometers long and two to three nanometers thick. This structure in turn is subdivided into a series of smaller tightly folded domains. It appears that each domain is responsible for one of fibronectin's binding functions with extended and flexible segments joining the domains.

The domain structure of fibronectin is also observed under the electron microscope and presents as slender, elongated molecule with regions of apparent flexibility (Engle, et al., 1981; Erickson, et al., 1981), although it can appear more globular under certain conditions (Koteliansky, et al., 1981). Variability also exists in the hydrodynamic radius, which is known to increase or decrease depending upon ionic strength (Alexander, et al., 1979). Generally, it can be interpreted from these studies that the fibronectins are highly flexible molecules that can expand

or contract depending upon the local environment.

The function of fibronectin is thought to be as an adhesive glycoprotein serving in preferential binding to host cells as well as to a variety of extracellular materials, such as collagen and the glycosaminoglycan regions of various proteoglycans (Yamada, et al., 1981; Vaheri and Mosher, 1978). Biologic macromolecules that interact with fibronectin include collagen and gelatin, fibrin and fibrinogen, heparin and heparin sulfate, hyaluronic acids, gangliosides, actin, DNA and transglutaminase (Mosesson and Amrani, 1980; Ruoslahti, et al., 1981). Fibronectin is also active in maintaining normal cell morphology, cell motility, chemotactic activity, embryonic differentiation and non-immune opsonization in the host defense system (Yamada, et al., 1981).

While fibronectin does function as a cell adhesive protein, it is not involved in the attachment of all cells. Chondrocytes do not produce fibronectin, although they do produce a somewhat analogous smaller protein termed chondronectin (Kleinman, et al., 1981). Epithelial cells do not synthesize fibronectin but produce a glycoprotein termed laminin for attachment of cells to the basal lamina (Yamada, 1981).

The movement of fibroblasts during wound healing is likely to be similar to other cells participating in wound healing (Abercrombie, 1961). The mechanisms involved in regulating cellular movement and leading to specific

macromolecular synthesis involves cell-to-cell and cell to substratum contacts mediated by cell surface molecules and diffusible molecules emitted by cells at, or remote from, the appropriate site (Hughes, et al., 1979). The activity of the substratum in cellular migration and orientation is termed contact guidance (Isenberg, et al., 1976; Heath and Dunn, 1978). The idea that adhesion between cells (or between a cell and a substrate) may be mediated by basically similar mechanisms was addressed after identification of the collagen dependent attachment factor, fibronectin (Klebe, 1974).

The characteristic that initially drew attention to plasma fibronectin was its presence in plasma precipitate which forms when plasma stands in the cold, hence, the name "cold insoluble globulin". The early methods that were used to isolate the protein made use of cryoprecipitation followed by separation of fibronectin by differential precipitation and ion exchange chromatography (Mosesson and Umfleet, 1970; Mosesson, et al., 1975; Mosher, 1975). Other methods of isolation of the soluble form of fibronectin rely upon fibronectin's affinity for gelatin-Sepharose (Engvall and Ruoslahti, 1977). A biologically active form of fibronectin has been isolated from the surface of cells by extraction with low concentrations of urea (Yamada and Weston, 1974; Yamada, et al., 1980). The procedure gently detaches the fibronectin from the plasma membrane without disrupting the membrane integrity. Other methods have been

used such as chelating agents, salts, detergents, guanidine and dithiothreitol but are much harsher agents resulting in cell lysis and a less effective means of extracting fibronectin (Olden, et al., 1979).

## B. Wound Healing

### 1. General considerations

When an organism is injured, a complex series of events is initiated in what is termed wound healing. Wound healing occurs by a process that the pathologists term repair (Anderson and Scotti, 1976) which may include one or more of these processes:

1. Resolution is the restoration of the involved tissue to a normal state which may occur when the injury is slight and there is only a degeneration of the perenchymal tissue without necrosis. After the inflammatory response subsides and the degenerated cells recover, the area is restored to normal.
2. Healing by granulation tissue or scar formation will occur if resolution is delayed or if an injury is severe enough to produce destruction of tissue.
3. Regeneration is the replacement of destroyed or lost cells by proliferation of cells of a similar type from adjacent living tissue.

The events following injury have been subdivided into several stages (Arey, 1936; Schilling, 1968; Ross and Benditt, 1961) which have been classified into the (1) inflammatory phase, (2) the proliferative or fibroblastic phase, and (3) remodeling phase. The inflammatory phase

commenses immediately following injury and is characterized by a typical acute inflammatory response. The cellular and humoral reactions which follow wounding serve several functions. Initial vasoconstriction mediated by vasoactive humoral factors aid in preventing blood and fluid loss from the ruptured blood vessels. Platelet adhesion with the formation of platelet plugs serves to seal the damaged ends of microvessels. A copolymer of fibrin forms a fibrin matrix and fibronectin (Kurkinen, 1980) acts as a substrate for the migration of fibroblasts and endothelial cells into the wound site.

Following the initial coagulation and microvascular permeability changes, leukocytes migrate into the extravascular wound. The cell which migrates the most rapidly is the polymorphonuclear leukocyte (PMN). These cells perform an important role in protecting the wound site against infection.

The next phase of repair is the fibroblastic proliferative phase and is characterized by the migration of fibroblasts from the uninjured tissues adjacent to the wound area and their subsequent proliferation. The proliferative stage does not become significant until the acute inflammatory stage begins to resolve. The actual induction of the fibroblasts into the wound is a complex process. Activation of resting cells, induction of migration through normal extracellular matrix towards the wound, migration on a fibrin-fibronectin meshwork and stimulation of mitosis

within the wound are all required for a fibroproliferative response. Once the fibroblasts are present within the wound, synthesis of extracellular matrix components including collagen, glycosaminoglycans, proteoglycans and structural glycoproteins must be controlled and coordinated in a fashion to result in successful repair.

During wound healing, fibroblasts migrate extensively through the extracellular matrix in a highly organized fashion. This directed migration of cells, chemotaxis, is in response to a chemical gradient. During this process, factors are released which stimulate the migration of neutrophils, fibroblasts, endothelial cells and smooth muscle cells to the site of injury where each cell type has a role in the tissue repair (Ross, 1968; Silver, 1980). Similarly, during bone induction, it is possible that osteoblasts (or their progenitor cells) may respond to signals that direct their migration and stimulate their attachment to areas of bone formation (Weiss and Reddi, 1980; Somerman, et al., 1982; Somerman, et al., 1983). Fibronectin provides the attachment which binds the cells to the extracellular matrix.

## 2. Special problems of periodontal wound healing

In discussing the healing of tissues in the oral cavity, there are two unique factors in the oral wound site. First, periodontal surgery cannot be accomplished in a sterile environment. The surgical site is bathed in

saliva and indigenous bacteria which have the ability to interact with the healing tissue site to potentially alter wound healing events. Second, the epithelial cell proliferation may outdistance connective tissue repair within a healing site and thereby prevent a functional regeneration of the periodontal ligament (Karring, et al., 1980; Magnussen, et al., 1985; Isador et al., 1985). Thus, if enhancement in the rate of connective tissue healing could be stimulated in the initial stages of wound healing by early guidance of the fibroblast to the area of injury, repair of the destroyed connective tissues may be possible.

### C. Bone Grafting Materials

Schallhorn (1977) has elaborated the objectives of bone augmentation in periodontally affected areas where substantial bone loss has occurred. These objectives include pocket reduction or elimination, restoration of the alveolar process, and regeneration of a functional attachment apparatus. In a search for substances that would regenerate lost tissues, three types of materials have been examined. First, there are materials which replace components of the lost periodontium by osteoconductive mechanisms (bone repair) which are initiated by already differentiated cells. Second, there is the mechanism of osteoinduction, whereby undifferentiated host cells (mesenchymal cells) differentiate and become committed to a bone formation development pathway (Takugi and Urist, 1982).

A third type is osteogenesis, which is the transplantation of osteoblasts or preosteoblasts into a new site where new bone is synthesized.

A variety of grafting materials have been examined: (1) alloplastics: implants of inert materials; (2) xenografts: tissue from different species; (3) autografts: tissue from the same individual; (3) isografts: tissues from the same species and genotype; and (4) allografts: tissue from the same species but a different genotype.

A variety of alloplastic materials have been used to treat osseous defects. Plaster of paris was among the first such materials to be used. It was believed that calcium salts would stimulate bone formation. However, there is no evidence that plaster of paris was effective in obtaining bone fill in periodontal defects (Schaffer and App, 1971). Ceramic calcium phosphates have also been used in the treatment of bony defects. All of the ceramic materials appear to be biocompatible, producing little or no apparent inflammation. These ceramics are believed to fill defects rather than promote bone formation (Nery and Lynch, 1978; Rabelais, et al., 1981; Levin, 1975). The ceramic material becomes encapsulated in a collagenous envelope (Mors and Kaminski, 1975; Froum, 1982) and again provides more of a filler thus reducing pocket depth via the formation of a long junctional epithelial attachment and a connective tissue adhesion (Froum, et al., 1982).

Xenografts have the advantage of being a readily

available graft material. However, the great concern with these materials is the immunological complications. Some graft rejection is almost certain and these materials have been reviewed with much caution (Mellonig, 1980). Biologic materials other than bone have been tried as graft materials due to their similarity to periodontal tissues. Sclera is one of these materials and was found to anchor periosteal and periodontal collagen fibers, but did not provide osteogenic stimulation. Evidence has also been presented that this material may impede wound healing (Moskow, et al., 1976). Collagen gels have also been tried in the treatment of periodontal osseous defects (Mellonig, 1980). Experiments indicate that the material was resorbed and replaced by bone, however, the osteogenic potential was never established.

Autografts (grafts using tissue transplanted from one site to another within the same individual) have been the most successful. Graft rejection is minimal and bone regeneration is enhanced (Mellonig, 1980).

The use of bone chips obtained during the surgical procedure utilizing osteoplasty and ostectomy has been suggested (Nabors and O'Leary, 1965) and modified to include smaller pieces of cortical bone combined with the patient's own blood into an osseous coagulum (Robinson, 1969). It appears that only small amounts of bone induction are available by any of these methods (Schallhorn, 1977). Bone Blend (Diem, et al., 1972) is a similar graft material. It

consists of a combination of cortical and cancellous bone obtained intraorally in combination with other procedures. Grafts of this type can be obtained from edentulous ridges (Hiatt and Schallhorn, 1973; Rosenberg, 1971), maxillary tuberosities (Kucaba and Simpson, 1978), healing extraction sockets (Soehren and Van Swol, 1979; Evian, et al., 1982), and surgically created healing sockets (Halliday, 1969). While the ability of intraoral cancellous bone marrow to regenerate bone is similar to that of iliac marrow grafts, the outcome is probably not as predictable. Intraoral cancellous bone marrow usually requires an additional surgical site, involves a variety of anatomical considerations, and provides a limited amount of donor material containing questionable amounts of hemopoietic marrow (Schallhorn, 1977).

Iliac autografts, composed of cancellous bone and hemopoietic marrow, appear to yield the most favorable and predictable results in correcting a variety of osseous defects, including furcation involvement (Schallhorn, 1967; Seibert, 1970; Schallhorn, et al., 1970; Dragoo and Sullivan, 1973). The material obtained from the iliac crest may be stored a variable amount of time by refrigeration or freezing (Schallhorn, et al., 1970). There are significant limitations to this material including additional surgical insult to the patient, logistical problems to be arranged coordinating the therapy, and the additional expense to the patient. When fresh marrow has been used, the incidence of

root resorption has increased (Schallhorn, 1977). However, this problem seems to be minimized by using refrigerated or frozen marrow or by frequent root curettage with reduction of inflammation at the surgical site (Dragoo and Sullivan, 1973).

While autografts provide many positive benefits for grafting procedures, the amounts of the material are limited and the procedures include invasive harvesting. An implant material that could provide the cancellous bone and hemopoietic marrow while obviating the disadvantages of autografts could prove to be a beneficial implant material. To this purpose, allografts or tissue from the same species, but dissimilar genotype have been pursued. Viable cancellous marrow is procured from "living cadavers" and stored cryogenically. This material has a high induction potential and may be obtained from a tissue bank. However, adverse immune responses are possible and patients must be immunologically cross-matched and typed (Schallhorn and Hiatt, 1972; Hiatt, 1971). Disease transmission can also be a problem which can be diminished through sterilization of the material, i.e. irradiation. However, this process may reduce the induction potential of the tissue (Schallhorn, 1977; Berdy, 1976).

Freeze-dried bone, first described by Hurt in 1968 as a material employed in osseous defects, has a diminished potential for antigenicity and disease transfer by virtue of freeze-drying and irradiation (Turner and Mellonig, 1981).

The bone induction potential is reasonably high for freeze-dried bone as demonstrated in numerous clinical studies, especially when the bone is combined with autogenous graft material (Mellonig, et al., 1976; Sepe, 1978; Sanders, et al., 1983). The osteogenic potential of freeze-dried bone has been further improved with decalcification. The decalcification process involves a series of steps including lipid extraction and decalcification after the bone has been lyophilized (Urist, 1965).

Subtle changes in the preparation and use of the grafting material can influence its osteogenic potential. For example, bone particles less than 75  $\mu$ m in diameter showed decreased osteoinductive properties when compared to larger particles (Syftstad and Urist, 1979; Reddi and Huggins, 1973; Reddi, 1973). The source of the bone used for implantation can also influence the osteoinductive influences. Diaphyseal bones such as femur, humerus, fibula and tibia have a higher osteogenic potential than bone from the pelvis, calvaria, scapula, or cartilage (Urist, et al., 1968; Reddi, 1973). The age of the donor bone as well as the age of the recipient influences bone inductive properties (Syftstad, 1982). The area of implantation also affects the osteogenic property. Implant sites in descending order of receptiveness are: bone and bone marrow, skeletal muscle, subcutaneous tissue, dermis, brain, lung, anterior chamber of the eye, testes, pancreas, and ovary (Urist, 1968). Sterilization also affects the

osteoinductive properties of the implanted material. Irradiation greater than  $1.0 \times 10^6$  rads decreases osteoinductive activity (Urist, et al., 1975); autoclaving completely destroys the activity (Urist, 1965; Urist, et al., 1967; Towle, et al., 1986). As a result, Mulliken, et al. (1984) utilizes ethylene oxide. However, a recent research article by Towle, et al. (1986) shows that bone induction in demineralized bone is markedly interfered with when ethylene oxide is used compared to electron beam bombardment ( $e^-$ ).

#### D. Rationale for use of decalcified bone

The rationale for decalcification relates to two functions in osseous regeneration. First, decalcification acts by providing a scaffold onto which new bone can grow. Second, osteoinductive factors are present in the graft material (Syfestad, 1984; Mulliken and Glowacki, 1980; Kaban and Glowacki, 1981; Reddi and Huggins, 1973) and are released or made accessible after partial removal of its mineral content.

#### E. Sequential events following implantation of DBP

Reddi and Anderson (1976) have described a reproducible cascade of events which occur when demineralized bone matrix is placed in subcutaneous sites resulting in the initiation of endochondral bone formation. At Day 1, the implant is a conglomerate of substances

consisting of graft particles, fibrin clot and polymorphonuclear leukocytes. By Day 3, the mesenchymal cells proliferate while the polymorphonuclear leukocytes have disappeared and fibroblast-like cells, surrounded by type III collagen, are shown in close approximation to the graft material. On Day 5, the fibroblast-like cells begin to differentiate into chondrocytes. Hyaline cartilage is produced by Day 7-8 and at Day 9, vascularization of the implant is seen concomitant with cartilage calcification. Osteogenic cells and osteoblasts are seen in the vicinity of sprouting capillaries on Days 10-11. Type I collagen is observed indicating bone development. From Days 12-18, the newly formed ossicles remodel resulting in the formation of hemopoietic bone. Type III collagen is seen only in association with these hemopoietic cells (Reddi, 1983).

These morphologic observations have been verified using a variety of biochemical parameters (Reddi and Anderson, 1976; Reddi, 1984). Day 1 is characterized by the release of peptides of fibronectin and an increase in cell motility. On Day 2 there is an initiation of protein and nucleic acid synthesis with a possible release of growth factors. On Days 3 and 5 there is a decrease in serum alkaline phosphatase activity. An increase of  $^{35}\text{SO}_4$  incorporation into proteoglycans corresponds with the differentiation of chondroblasts. Type II collagen synthesis and cartilage specific proteoglycan synthesis are observed on Day 7. The serum alkaline phosphatase increases, showing its first peak

at this time, corresponding to the presence of chondrocytes with the synthesis and secretion of matrix. Type IV collagen synthesis, increase in  $^{45}\text{Ca}$  incorporation, and detection of factor VIII in blood vessels are observed on Day 9. A second peak of alkaline phosphatase activity occurs and corresponds to osteoblast activity and bone formation. This alkaline phosphatase peak is higher than that seen on Day 7. Bone proteoglycan synthesis and type I collagen synthesis are observed. Days 12-18 are characterized by an increase in lysosomal enzymes (acid phosphatase, aryl sulfatase and proteases). An increase in iron incorporation into heme and type III collagen synthesis corresponding to bone marrow differentiation is seen on Day 21.

#### F. Rationale for use of fibronectin

In view of the fact that the implanted material is predominantly collagenous, Weiss and Reddi (1980) examined the presence of fibronectin during early matrix-cell interactions. Upon implantation, the matrix binds endogenous fibronectin from 3-18 hours after implantation (Reddi, 1984) which may constitute an important initial event for cell attachment to the matrix. The initial orientation of cell surface to matrix is by electrostatic forces but it is likely that the collagen-fibronectin interaction aids in bringing the inductor in contact with focal cell surface receptors (Reddi, 1984).

Predictably, periodontal therapy has not obtained significant amounts of new bone following implantation of bone grafting material. Studies using animal and human models have presented histologic evidence of new attachment (including cementogenesis and connective tissue attachment) following demineralization of scaled or planed root surfaces (Albair, et al., 1982; Cole, et al., 1980; Nilveus and Egleberg, Register and Burdick, 1975; Register and Burdick, 1976). Boyko, et al. (1980) investigated the effect of root demineralization on cell attachments in vitro and found greater attachment of cultured fibroblasts to treated teeth than to non-demineralized controls. These authors concluded that the demineralization of root surface collagen created a substrate to which fibronectin (synthesized by the fibroblast) could attach. Terranova, et al. (1981), have found that the attachment of fibroblasts to root surfaces was greatly increased in the presence of additional fibronectin.

In studying the fibronectin binding capacity of demineralized bone and dentin, Pearson (1986) found that endogenous serum contained more fibronectin than the binding capacity of bone receptors. For 50 mg of demineralized rat bone, 70 ug of fibronectin was bound in an in vitro situation (1.4ug fibronectin/ mg demineralized rat bone matrix). However, the wound healing role of fibronectin bound to DBP prior to implantation has not been addressed. It may be possible, by the addition of exogenous serum

fibronectin, to alter the wound healing sequence by allowing fibronectin to be present at the wound site from the first moments of implantation and therefore increase bone formation.

#### G. Statement of Problem

Weiss and Reddi (1980) have shown the presence of endogenous fibronectin from 3-18 hours after implantation of demineralized bone into subcutaneous sites. Since the collagen-fibronectin interaction appears to be an important initial factor in the chemoattraction and attachment of fibroblasts, it would be of interest to determine if the addition of exogenous fibronectin would have a beneficial effect on the mesenchymal tissue differentiation when added to demineralized bone matrix prior to implantation. Pearson (1986) has shown that the amount of fibronectin in serum far exceeds the binding capacity of demineralized bone. However, the time frame of when the fibronectin is available at the wound site may have some influence on the rate of wound healing. Fibronectin binding to the collagen of the demineralized bone matrix prior to implantation of the the demineralized bone would allow fibronectin to be present from the first moment of implantation. The initial enhancement of connective tissue healing with fibronectin could be beneficial in the treatment of periodontal osseous defects since the competition between connective tissue healing and epithelial migration is of primary importance

and early enhancement of connective tissue proliferation could result in determining the tissue type present.

The purpose of this research is to determine the response of mesenchymal tissues when demineralized bone matrix, which has been treated prior to implantation with either phosphate buffered saline (PBS) or PBS with fibronectin, is placed into subcutaneous sites of Long Evans rats.

### III. METHODS AND MATERIALS

#### A. Experimental samples

##### 1. Preparation of Long Evans Rat Bone

Long bones including the femur, fibula and tibia from 150 male Long Evans rats were obtained frozen from Hilltop Lab Animals, Inc. (Scottsdale, Pennsylvania). The bones were stored at -20 degree C until used. The bones were from otherwise healthy rats which, in no way, had altered systemic health. Soft tissues were removed down to and including the periosteum. The epiphyses were removed and an endodontic file was used to remove the bone marrow and endosteum while the bone was irrigated with ultrapure water. After washing the bone segments five times with ultrapure water, the segments were defatted by extraction for 1 hour in 100% ethanol and dehydrated for 1 hour in anhydrous ether. The organic solvents were removed by decantation and evaporation.

The defatted, dehydrated bone segments were ground into a powder using a Spex freezer mill. The powder was sifted with mesh screens to separate the particles into the following sizes: greater than 425 um, 425-300 um, 300-150 um, and less than or equal to 150 um. The bone particles of the size 150-425 um were used in this study.

## 2. Demineralization of Long Evans Rat Bone

Long Evans rat bone powder was demineralized using the method described by Kaban and Glowacki (1981). Bone powder (particle size 150um-425um) was demineralized using 0.5 M HCl (25 ml/g bone) for 3 hours at room temperature. Acid and dissolved minerals were washed away by copious changes of ultrapure water at 4 degree C until neutral pH was achieved. Following the demineralization, the bone was dehydrated by sequential extractions for 1 hour each in 100% ethanol and anhydrous ether. The solvents were removed by decantation and evaporation. Eight hundred increments of 30mg each of the demineralized bone powder was weighed using a triple beam balance and each increment was placed in individual 1 ml tuberculin syringes which had the restricted terminal section removed which provided a barrel with plunger apparatus for the application of the bone powder. The syringes with the 30mg of bone powder were packaged and sterilized using ethylene oxide for a period of 2 hours at 130 degrees F and placed in a chamber for 12 hours with mechanical aeration. The packages of sterilized bone powder were then stored under a laminar flow hood for a period of at least 48 hours to ensure the complete evaporation of the gas.

## 3. Preparation of Fibronectin

Rat fibronectin was prepared from Long Evans rat plasma obtained in frozen form from Hilltop Lab Animals,

Inc. (Scottsdale, Pennsylvania) and purified by cyanogen bromide activated gelatin sepharose chromatography in the laboratory of Robert Klebe, Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio (Klebe et al. 1980). Cyanogen bromide activated gelatin sepharose in a sintered glass funnel was washed sequentially with 0.9% saline (1 time), 8M urea plus 50mM sodium citrate (2 times), 0.9% saline (3 times), and standard phosphate buffered saline (1 time). After weighing, the cyanogen bromide packed gel was placed in a beaker. Eighty-eight ml of rat plasma were added to 44 ml of packed gel. This was stirred occasionally for 30 minutes in a cold room at 4 degrees C. The gel was then washed with phosphate buffered saline containing 10 mM sodium citrate, pH 7.2, until the gel was white. Twenty-two ml of 8M urea containing 50mM sodium citrate, pH 4.7, were added and allowed to percolate through the gel and collected in a clean flask using a sintered glass funnel. The sample was placed under vacuum until dried. A second twenty-two ml aliquot of 8M urea plus 50mM sodium citrate was added to the gel and collected and dried in the same manner. The sample obtained was dialyzed as 44 ml of eluate against standard phosphate buffered saline. The cyanogen bromide activated gelatin sepharose column was then washed with standard phosphate buffered saline to prepare it for the next use. The procedure was repeated two more times until 132 ml of eluate were dialyzed against standard phosphate buffered

saline. The plasma fibronectin concentration was determined using the direct enzyme linked immunosorbent assay (ELISA) comparing the rat plasma fibronectin to a fibronectin standard of known concentration. Three hundred ml of eluate were found to have 26.95 mg of fibronectin or 80 ug of fibronectin/ ml.

#### 4. Preparation of the Implants:

Each implant consisted of 30 mg of demineralized bone powder which was placed in a tuberculin syringe and sterilized with ethylene oxide gas. Eight hundred separate implants were prepared in the carrier syringes. Twenty four hours prior to the actual implantation of bone powder into the rats, the syringes with the bone powder were divided into two equal groups: the first group (control) of 400 syringes had 0.7 ml of PBS added to each 30 mg increment of bone powder. The second group (experimental) of syringes with the demineralized bone powder had 0.7 ml of PBS containing fibronectin added (ie. 56 ug of fibronectin per implant). The two groups were identified as group A (yellow) and group B (blue) by a person unrelated to the project who identified which group had the saline alone and which group had the fibronectin. This information was placed in a sealed envelope leaving the identity of the groups unknown to the research examiner. The syringes were placed in a 4 degree C cold room for 24 hours to enable the fibronectin to bind to demineralized bone powder. Thirty

minutes prior to implantation, the excess fluid was removed by expulsion, leaving the demineralized bone powder pellet ready for implantation.

B. Surgical Procedure:

1. The Effects of Fibronectin

One hundred and eight-eight Long Evans rats were divided randomly into 2 groups. All the rats were normal adult males approximately 2 months of age and weighing 200-224 grams (Harlan Sprague Dawley, Indianapolis, Indiana). One group was designated to receive 4 implants apiece of the demineralized bone powder from the yellow group and the second group was designated to receive 4 implants apiece of the demineralized bone powder from the blue group. Each rat obtained adequate anesthesia from an intraperitoneal injection of sodium pentobarbital (40mg/kg). Each rat's thorax and abdomen were shaved using electric clippers. Aseptic, surgical preparation of the implantation sites was accomplished prior to surgical incisions. One sharp incision through the skin to the superficial fascia was placed starting on the midline beneath the sternum and extending caudally 1/2 inch. Four pouches were prepared, two on each side of the incision by use of blunt dissection laterally from the incision into the subcutaneous fascia. It was into these four pouches that the implants were placed. The incisions were closed with 4-0 gut suture. Group B rats (blue) had their left ear notched. When recovered, the

animals were returned to standard caging (5 rats per cage).

2. The Effect of Recipient Age and Dry  
Implantation of Demineralized Bone Powder.

Four young (3 weeks old, approximately 120 grams) Long Evans rats were obtained from Harlan Sprague Dawley (Indianapolis, Indiana). Each of the rats obtained adequate anesthesia from an intramuscular injection of a combination of ketamine, xylosine and acetylpromazine. The anesthetic mixture is sufficient to allow 0.1 ml to anesthetize a 250 gram rat. Therefore, 0.05 ml of the anesthetic solution was used to obtain adequate anesthesia for the 120 gram rats used in this section of the study. Surgical preparation and incisions were accomplished in the same manner as the first surgical procedure. Each rat received four implants placed in a manner similar to the first surgical procedure. However, in contrast to the first surgical procedure, the demineralized bone powder was placed in a dry powder form in two of the implants in each rat. The other two implants in each rat were incubated in PBS for 24 hours prior to implantation in a manner similar to the first surgical procedure.

C. Experimental Technique

1. Technique #1

Seven rats each from Group A and Group B were sacrificed on days 1, 3, 7, 9, 11, 14, 18, 21 and 28 using T-61 euthanasia solution. The harvested implants from these

animals were placed in plastic vials and frozen at -70 degree C until biochemical analysis could be completed. Only the implants that were well formed in a discrete mass were used in the analysis. Five rats from each group were sacrificed in a similar manner on days 3, 7, 9, 11, 14, 18, 21 and 28 with the implants placed in 10% buffered (sodium phosphate monobasic and sodium phosphate dibasic) formalin to be decalcified and prepared for histologic examination.

## 2. Technique #2

To test whether the recipient age or the use of saline to wet the implant could have resulted in fibrous encapsulation rather than bone induction, two of the four rats were sacrificed (using CO<sub>2</sub> asphyxiation) at day 10, and the remaining two rats at day 21. The harvested implants were prepared for histologic examination.

## D. Biochemical Analysis

Seven rats were sacrificed on each designated harvest day. Each rat had four areas where DBP had been implanted. Thus, there was a possibility of having 28 implants on each harvest day for biochemical analysis. However, only well formed implants were used. Therefore, a random selection of 12 implants from each harvest day were chosen for biochemical analysis with any remaining implants being stored at -70 degrees C for further evaluation. The implants obtained for biochemical analysis were divided into

three groups containing 4 implants each. To each group, composed of 4 implants, ultrapure water was added to make a 2 ml volume. The 4 implants were then homogenized using a Tekmar Ultra Turrax Tissue Homogenizer. Using an automatic pipette, the homogenized implants were subdivided into 200 ul aliquots for alkaline phosphatase analysis, 100 ul aliquots for Lowry protein analysis, and three 100 ul aliquots for determination of the mineral content.

#### 1. Protein Concentration

Protein concentration was measured by using the Lowry protein assay (1951) using 0.1% SDS in the assay mixture. Bovine serum albumin was used as the standard. The data is expressed in mg protein/implant. This data is used as the base line for other analyses (ie. enzyme activity and mineral content).

#### 2. Mineral Content

One hundred microliter aliquots of sample were dried in preweighed crucibles at 100 degrees C and weighed using an electronic microbalance (Mettler Instruments, CH-8606 Greifensee, Switzerland). The material was ashed at 700 degrees C, cooled to room temperature, and weighed. The organic content was calculated as the "100 degree weight" minus the "700 degree weight". The ash was dissolved in 0.1 ml 12 N HCl and diluted to 2.0 ml with ultrapure water. Two aliquots were used to measure calcium

(Willis, 1961) and magnesium (Willis, 1961) by atomic absorption spectrophotometry. The inorganic phosphate was measured colorimetrically (Fiske and SubbaRow, 1925).

### 3. Alkaline Phosphatase Activity

The alkaline phosphatase activity is measured as an indicator of cartilage and bone mineralization. The alkaline phosphatase activity was determined (Stagni et al., 1979) using para-nitrophenol phosphate (PNPP) as the substrate. Specific activity was assayed at pH 10.2 and expressed as umoles para-nitrophenol formed per mg protein per hour.

### E. Histology

The harvested implants were placed in 10% neutral buffered (sodium phosphate monobasic and sodium phosphate dibasic) formalin for approximately 72 hours to fix the tissue. The implants were then placed in formic acid - sodium citrate with agitation for approximately 5 days. Decalcification was confirmed by x-ray. The implants were then rinsed with water and processed through formalin, graded alcohols, xylene and paraffin. The decalcified implant were then embedded in paraffin, sectioned and stained. The implants were divided into approximately six equal parts and from each part, 3 sections were obtained. Sections were cut 5-6 microns in thickness and stained (Manual of Histologic Staining Methods of the Armed Forces

Institute of Pathology, third edition, 1968) using hematoxylin and eosin (H & E), Masson's trichrome (utilizing aniline blue solution) and Safranin-O.

Hematoxylin and eosin was used as a standard staining method, whereas the Masson's trichrome and Safranin-O are special stains used to more specifically stain for the connective tissue elements. The Masson's trichrome stains the cytoplasm, muscle fibers and intercellular fibers red while the collagen is stained blue. The Safranin-O stain is used as a special stain in studying bone. Chondrotin sulfate and cartilage are stained a bright red while the demineralized bone, red blood cells, and other connective tissue elements are stained a gray-green. Bone is stained a red-violet.

#### F. Statistical Analysis

Numerical data obtained from the biochemical analysis were analyzed using a two way analysis of variance (ANOVA) for days and groups with a Systat (Systat, Inc. 1985) computer package. Fisher's Least Significant Difference was used as the post hoc test at  $p \leq 0.05$ . In analyses where no differences were found between groups, a one way analysis of variance was done between days and the LSD was recalculated.

#### IV. RESULTS

##### A. Histologic Characterization

###### 1. Effect of fibronectin in PBS

Based on histologic characterization of random sections, no differences in the tissue response to the DBP with PBS or to DBP in PBS containing serum fibronectin were evident. Sections from both experimental groups are similar histologically, regardless of which staining methodology was used. The tissues were characterized by the following observations.

Day 3: A loose connective tissue with a mild lymphocytic infiltrate was located around the periphery of the DBP. The tissue between the implanted DBP particles also consisted of a loose edematous connective tissue with a mild infiltrate of polymorphonuclear leukocytes (PMNs) (Plate 1).

Day 7: The loose connective tissue at the periphery of the harvested implants was less vascular than that seen in the Day 3 specimens. The connective tissue stroma present between the implanted DBP particles was more vascular and demonstrated fewer PMNs than Day 3 specimens, suggesting a diminution of the acute inflammatory response to the implantation. The connective tissue surrounding the implant contained endothelial cells and a few multinucleated giant cells were observed.

Day 9: The tissue periphery was still composed of a

loose connective tissue stroma. The tissue between the DBP particles demonstrated increasing vascularity. Little inflammatory infiltrate and few multinucleated giant cells were present.

Day 11: An increased cellularity, composed primarily of endothelial cells, was present between the DBP particles.

Day 14: The connective tissue between the DBP particles was more fibrous than earlier specimens and appeared hyalinized in some areas with other areas exhibiting a moderate infiltrate of lymphocytes. Dense vascularity continued to be present in most of the connective tissue and an increase in the multinucleated giant cells was observed (Plate 2).

Day 18: There was a marked increase in the number of lymphocytes as well as an increase in the fibrous connective tissue. This tissue was characterized by a dense fibrillar network. The fibrillar network was more evident where the cellular infiltrate was less. The hyalinized and woven bone-like regions were sparse.

Day 21: The histologic appearance of the tissues were similar to that of day 18. However, numerous multinucleated giant cells were observed adjacent to the DBP particles. There was no longer any evidence of hyalinized appearing areas.

Day 28: The DBP particles were surrounded by a dense collagenous network characteristic of fibrotic encapsulation of the individual DBP (particles) (Plates 3 and 4).

## 2. The effect of preincubation in PBS

The fibrous encapsulation of the DBP particles was present in specimens obtained when the DBP was implanted in a 'wetted' form, whether the rats were 2 months old or 3 weeks old. Therefore, the difference in the ages of the recipient rats, within the confines of this investigation, did not affect the results. In addition, the DBP preparation did contain active bone inductive potential. Tissue implanted with dry DBP particles harvested at Day 10 (Plates 5 and 6) were histologically characterized by evidence of residual cartilage and the formation of bone matrix (ie., osteoid). By Day 21, bone was present with evidence of remodeling (Plates 7 and 8). However, specimens harvested at Day 10 from animals implanted with DBP preincubated in PBS for 24 hours exhibited no evidence of cartilage. The tissue between the DBP particles exhibited moderate vascularization with a slight lymphocytic inflammatory infiltrate. By day 21, the tissue had a similar appearance to that which was described previously for the specimens harvested from day 21 in the group that was implanted with DBP in PBS only or with PBS and fibronectin. The DBP particles were surrounded by a dense fibrous network, characteristic of fibrosis.

Plate 1. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 3  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER INCUBATED IN PHOSPHATE BUFFERED SALINE  
(PBS) CONTAINING FIBRONECTIN.

The demineralized bone powder (DBP) particles are  
surrounded by a loose, edematous connective tissue. Arrows  
point to polymorphonuclear leukocytes (Safranin-O stain,  
X210).

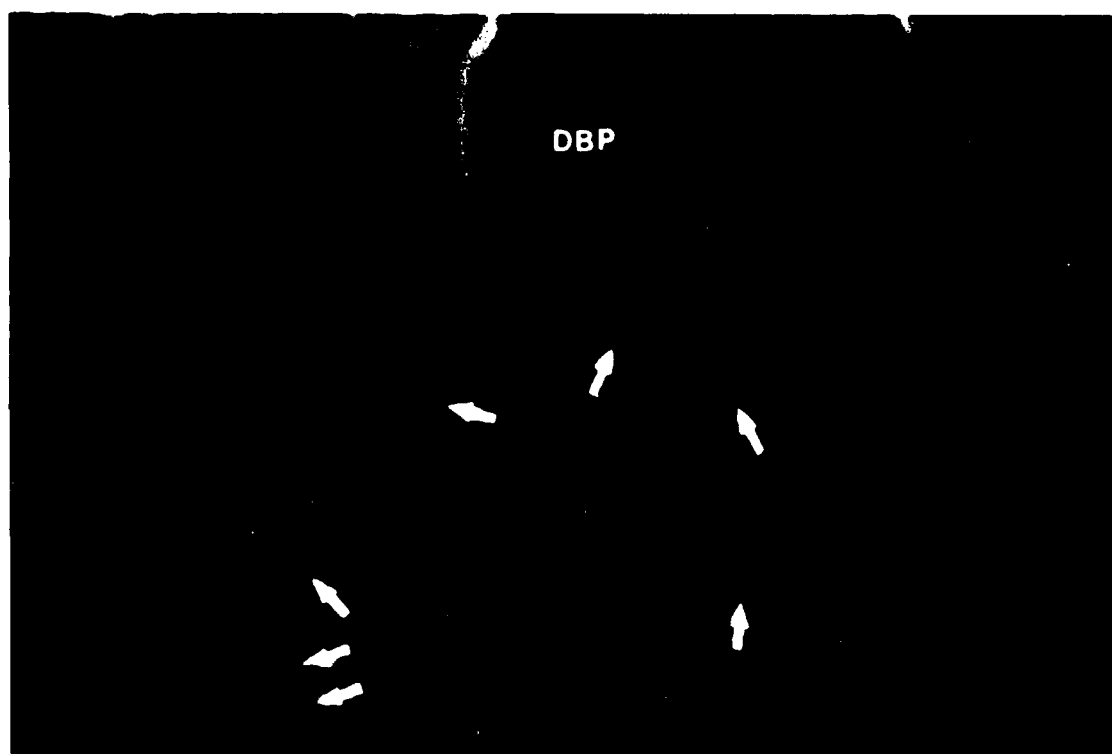


Plate 2. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 14  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER INCUBATED IN PHOSPHATE BUFFERED SALINE  
(PBS).

The demineralized bone powder (DBP) particles are  
surrounded by a matrix (arrows) that has the appearance of  
early woven bone (Safranin-O stain, X50).

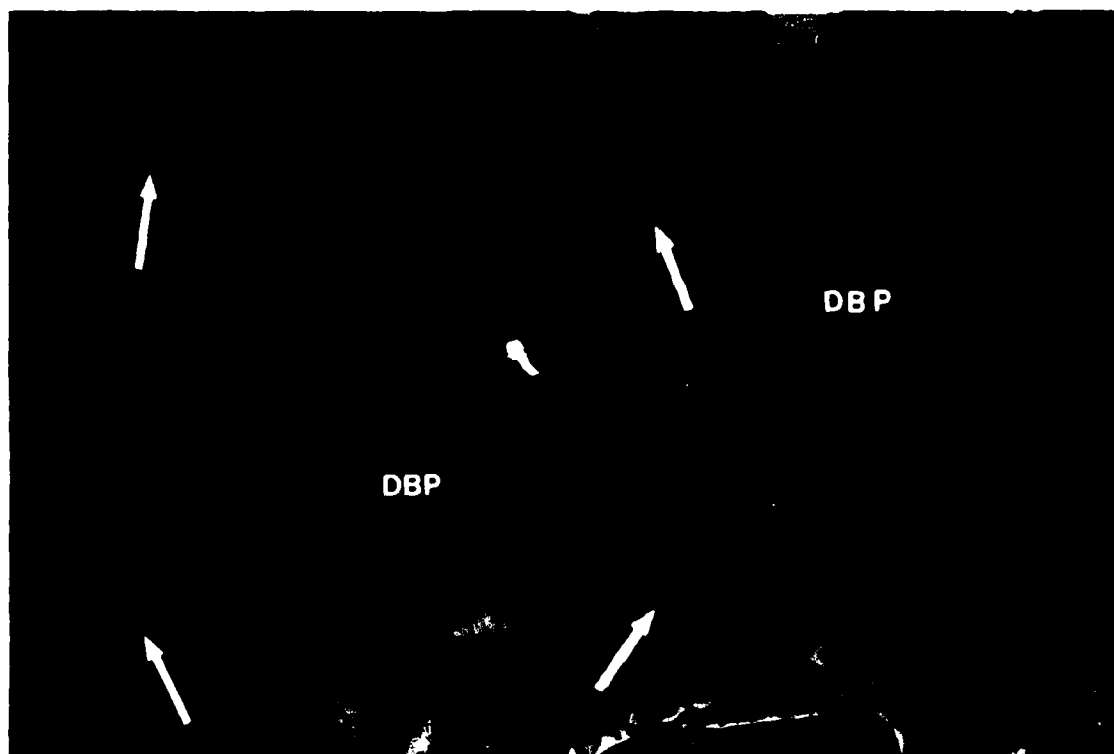


Plate 3. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 28  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER INCUBATED IN PHOSPHATE BUFFERED SALINE  
CONTAINING FIBRONECTIN.

The demineralized bone powder (DBP) particles are  
surrounded by a dense fibrillar network (arrows) (Safranin-O  
stain, X100).

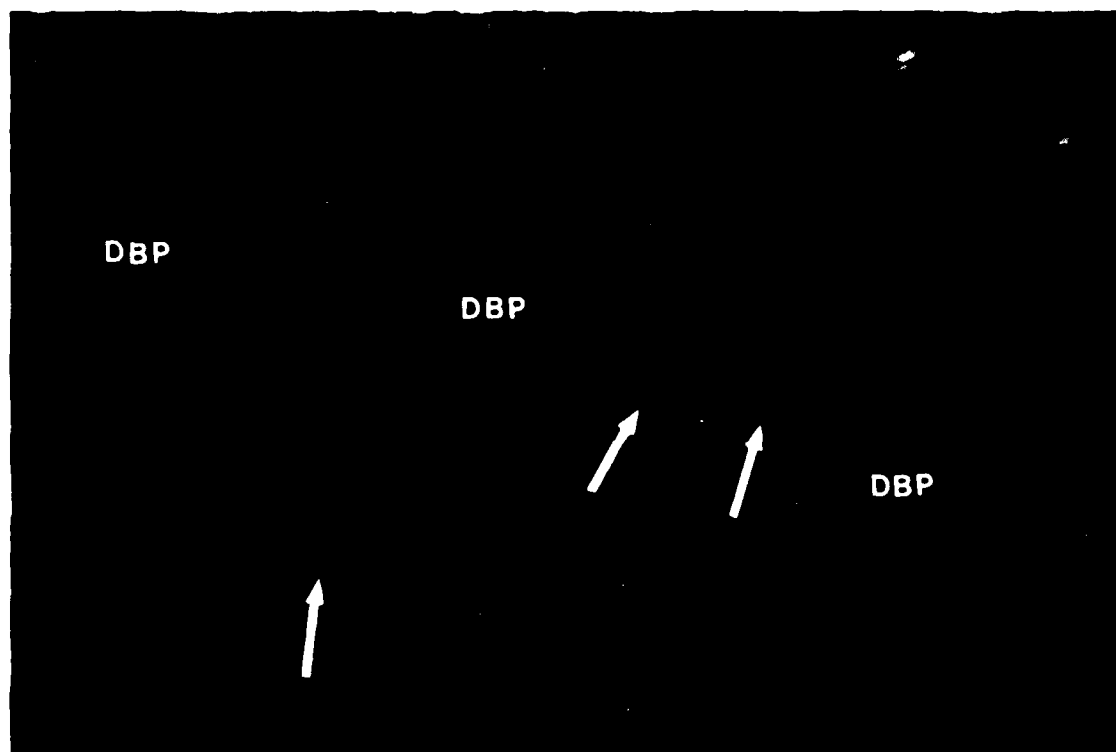


Plate 4. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 28  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER IN PHOSPHATE BUFFERED SALINE.

The demineralized bone powder (DBP) particles are  
surrounded by a dense collagenous network (arrows) (Masson's  
Trichrome stain, X210).

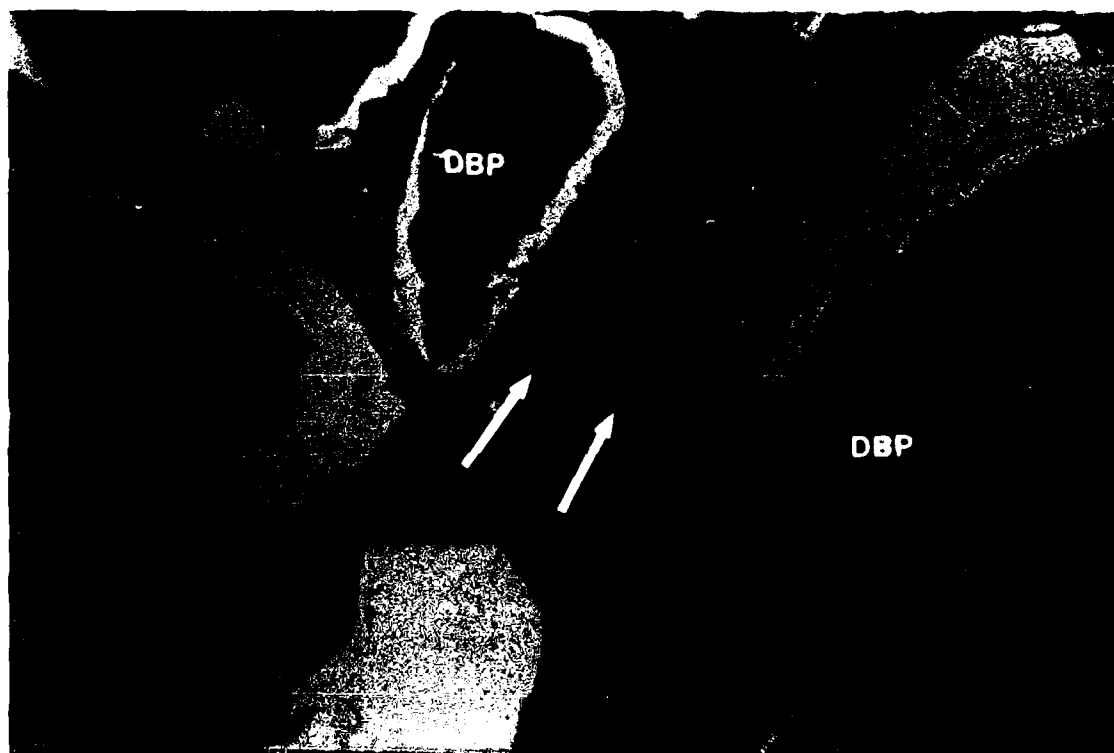


Plate 5. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 14  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER (DBP) PLACED AS DRY PARTICLES.

The demineralized bone powder (DBP) particles are  
surrounded by cartilage (C) and matrix (M) (Safranin-O  
stain, X100).

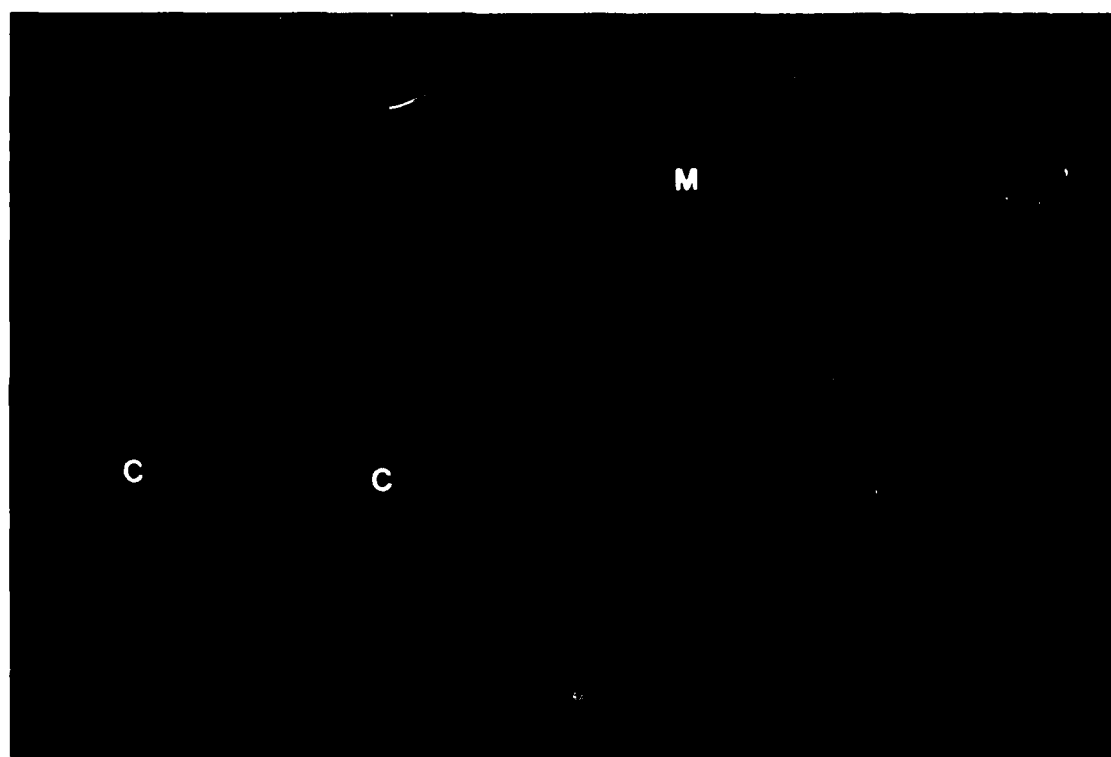


Plate 6. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 14  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER (DBP) PLACED AS DRY PARTICLES.

The demineralized bone powder (DBP) particles are  
surrounded by cartilage (C) and new bone matrix (NB)  
(Safranin-O, X210).

NB

C

DBP

DBP

Plate 7. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 28  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER (DBP) PLACED AS DRY PARTICLES.

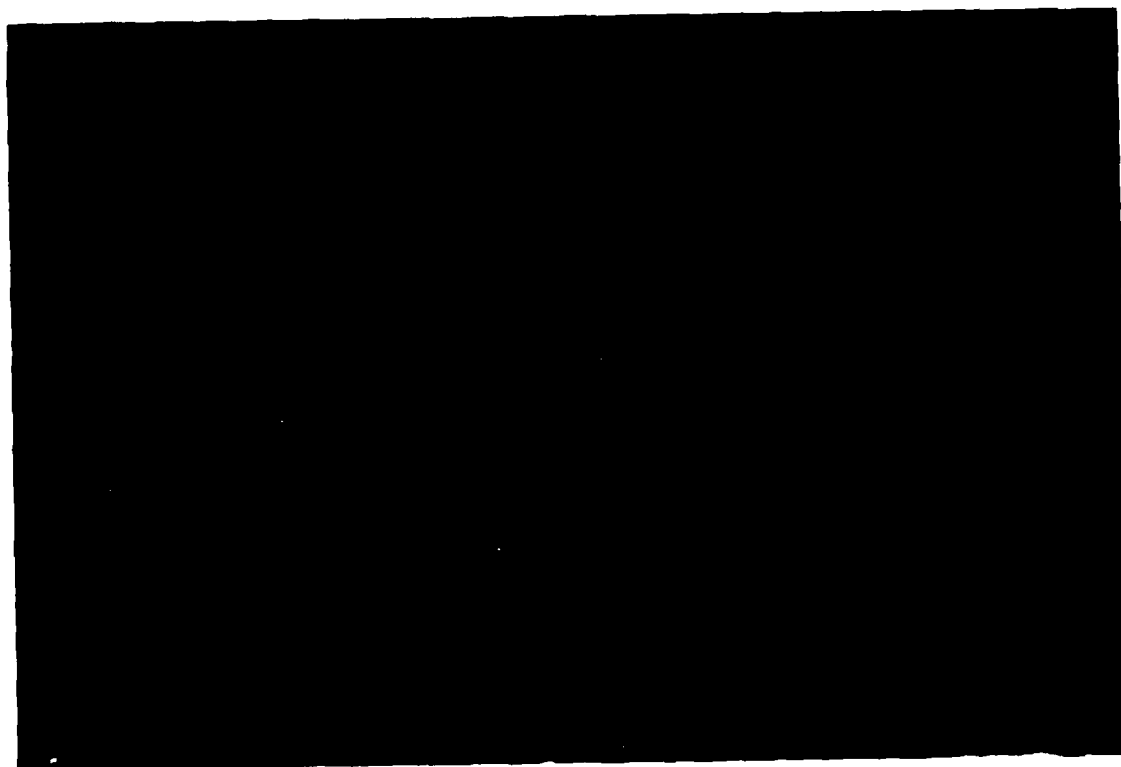
The demineralized bone powder (DBP) particles are  
surrounded by new bone matrix and cartilage (C) (Safranin-O  
stain, X100).

DBP

C

Plate 8. MICROGRAPH OF MESENCHYMAL TISSUE HARVESTED AT 28  
DAYS POST IMPLANTATION OF DEMINERALIZED BONE  
POWDER (DBP) PLACED AS DRY PARTICLES.

The bone is in transition. The red-purple stain  
indicates areas of newly mineralized bone (Safranin-O stain,  
X210).



## B. Biochemical Analysis

After all the data were collected and compiled, the code which identified the groups in which the DBP was implanted with PBS only or with PBS containing fibronectin was broken and it was determined that the yellow group (Y) was the DBP implanted with fibronectin in PBS and the blue group (B) was the DBP in PBS only. In the Appendix, the data are presented by post implant day ( 1, 3, 7, 9, 11, 14, 18, 21, or 28), the group (Y or B), and the division of the implants from within the group (A, B, or C). Thus, the notation "Day 1-Y-A" would designate the implant harvested 24 hours after implantation (Day 1); the implant was from group Y (the yellow group or the fibronectin with PBS); and, it was from sample A (a division of the implants from within the Day 1 yellow group). The results will also be presented in context with the broken code and thus, the Y group will also be referred to as F (PBS with fibronectin) and the B group will be referred to as S (PBS only).

### 1. Protein Content

There was considerable fluctuation in the protein content of the implants (Figure 1). No statistically significant differences were found in the protein content of the harvested implants when 'wetted' implants with PBS alone or PBS combined with fibronectin were compared using the two way analysis of variance (Figure 1 and Appendix). Differences in protein content were

observed with time; therefore, calculations were performed using a one way analysis of variance to determine the differences in protein content between days. The data revealed the protein content was statistically significantly higher ( $p \leq 0.05$ ) in the implants harvested on Day 1 than on any other day except Day 11. The protein content of the implants harvested on Day 28 was statistically ( $p \leq 0.05$ ) lower than that found on any other harvest day except Day 18.

Basically, there appears to be a relatively high protein content initially. The implants exhibit a peak in protein content on Day 11 which is significantly greater ( $p \leq 0.05$ ) than the preceding harvest day (Day 9) or the following harvest day (Day 14). The protein content of the implants is statistically ( $p \leq 0.05$ ) lower at Day 28 than at any other day except Day 18.

## 2. Alkaline Phosphatase Activity

Initially, specific activity of alkaline phosphatase is relatively high on Day 1 in both the fibronectin group and the saline only group of implants (Figure 2). The specific activity of the harvested implants decreases significantly ( $p \leq 0.05$ ) from Day 1 to Day 3. The mean specific activity of the implants peaks on Day 14. There is a significant difference ( $p \leq 0.05$ ) between the specific activity measured on Day 14 and the activity measured for any other harvest day. The specific activity

of the implants harvested on Day 14 is higher than any other harvest day except the implants harvested on Day 1.

In summary, there appears to be a high alkaline phosphatase activity initially with a drop in activity seen at Day 3 and continuing until Day 14. On Day 14 there is a 'peak' in activity which is statistically significantly ( $p \leq 0.05$ ) greater than that seen in other samples on all other harvest days except Day 1.

### 3. Ash Content

The ash content (Figure 3) increased in tissues which received the DBP either incubated with PBS alone or PBS with fibronectin. The greatest amount of ash content was observed at Day 21 in the tissue from animals receiving the DBP particles incubated in PBS combined with fibronectin. This response was greater than in the implants harvested on Days 1F, 1S, 3S, 3F, 7F, 18S, 28S and 28F ( $p < 0.05$ ). At Day 28, however, the ash was replaced by organic material, especially in the fibronectin treated implant group. In fact, these samples had the lowest ash content of all the samples measured and were statistically ( $p \leq 0.05$ ) lower than those samples harvested on Days 21F, 21S, and 11F (Appendix).

#### 4. Mineral Ion Content of Ash

##### a. Magnesium

The magnesium (Mg) content of the ash did not significantly change either between groups or among samples harvested on different days. The means ranged from approximately 1.04 to 3.89 ug Mg/ mg ash (Figure 4 and Appendix).

##### b. Calcium and Phosphate Content

A two way analysis of variance determined there was a difference between the samples harvested on different Days but no difference between the PBS only and the PBS containing fibronectin groups. However, when a one way analysis of variance was accomplished to establish any differences as a function of time, there was no statistical difference. The mean calcium content ranged from approximately 28 to 82 ug Ca/ mg ash. The mean phosphate content ranged from approximately 16 to 54 ug Pi/ mg ash (Figures 5, 6 and Appendix).

##### c. Calcium: Phosphate Ratio

Statistically, the ratio of calcium to phosphate was not significantly different between the groups nor did it change among samples harvested on different days. The means ranged from approximately 1.3 to 2.3 (Figure 7 and Appendix).

Figure 1. PROTEIN CONCENTRATION OF HARVESTED DEMINERALIZED BONE POWDER (DBP) IMPLANTS.

DBP incubated in phosphate buffered saline (PBS)  $\pm$  fibronectin was implanted into the thorax of rat subcutaneous fascia and harvested at 1, 3, 7, 9, 11, 14, 18, 21 and 28 days post implantation. Protein content was determined by the method of Lowry on homogenates of 12 implants randomized as 3 samples. Data are presented as means  $\pm$  S.D.; N=3. The data for Day 1 are presented as an average of 2 samples.

# PROTEIN CONTENT

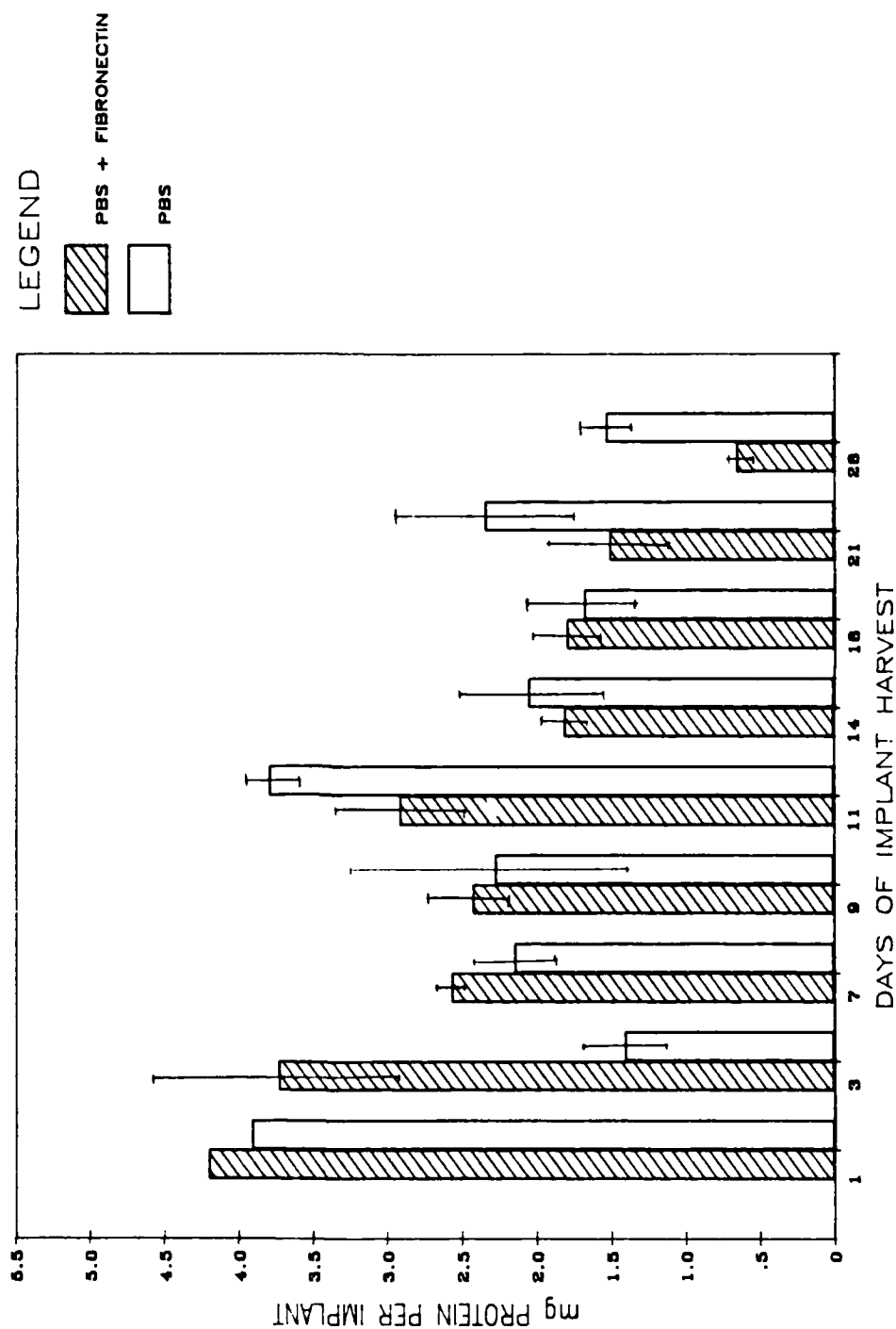


Figure 2. CHANGE IN ALKALINE PHOSPHATASE SPECIFIC ACTIVITY  
IN RAT SUBCUTANEOUS FASCIA IMPLANTED WITH  
DEMINERALIZED BONE POWDER (DBP).

DBP was incubated with phosphate buffered saline (PBS)  $\pm$  fibronectin and implanted in the subcutaneous fascia of the rat thorax. Twelve implants each were harvested at 1, 3, 7, 9, 11, 14, 18, 21 and 28 days and randomized into 3 groups and homogenized. The specific activity of alkaline phosphatase was determined as umoles of phosphate hydrolyzed from p-nitrophenol/mg Lowry protein/minute. Data are presented as an average of two samples for day 1 and the mean  $\pm$  S.D. of 3 samples for all other harvests.

# ALKALINE PHOSPHATASE SPECIFIC ACTIVITY

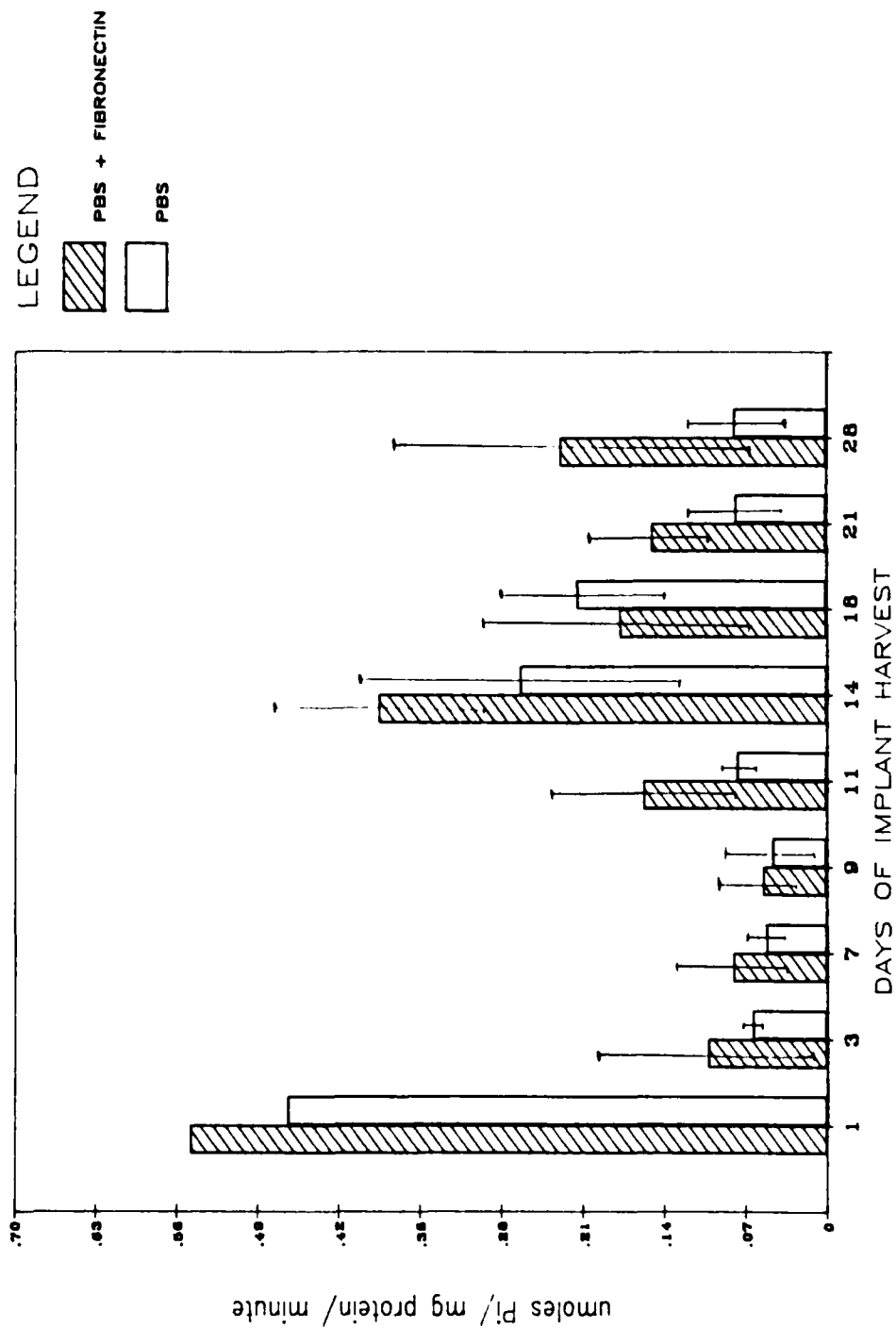


Figure 3. CHANGE IN THE INORGANIC CONTENT OF MESENCHYMAL TISSUE FOLLOWING IMPLANTATION OF DEMINERALIZED BONE POWDER (DBP) IN THE SUBCUTANEOUS FASCIA OF THE RAT.

DBP was incubated with phosphate buffered saline (PBS)  $\pm$  fibronectin and implanted in the subcutaneous fascia of the rat thorax. Twelve implants each were harvested at 1, 3, 7, 9, 11, 14, 18 21 and 28 days and randomized into 3 groups and homogenized. The dry weight was obtained when the sample was heated to 100 degrees C and the ash weight obtained after heating the sample to 700 degrees C.

Data are presented as an average percent of ash weight (inorganic) to the total dry weight. Day 1 samples are expressed as an average of 2 samples and all other harvests are expressed as the mean  $\pm$  S.D. of 3 samples.

# PERCENT INORGANIC COMPONENT OF THE HARVESTED IMPLANTS

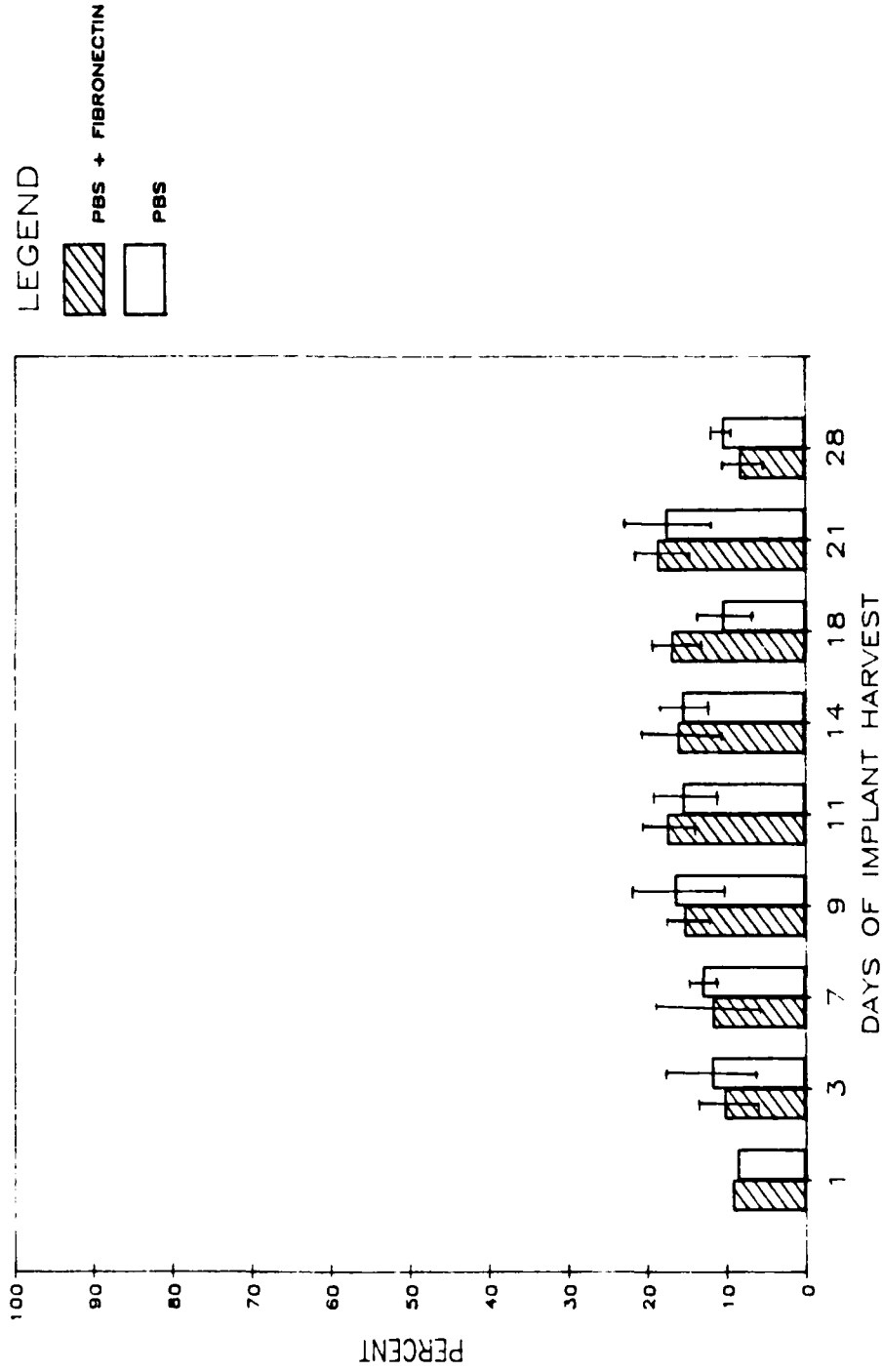


Figure 4. CHANGES IN THE MAGNESIUM CONTENT OF THE ASH OF MESENCHYMAL TISSUE INDUCED BY IMPLANTATION OF DEMINERALIZED BONE POWDER (DBP)  $\pm$  FIBRONECTIN IN THE SUBCUTANEOUS FASCIA OF THE RAT THORAX.

The ash sample was obtained from mesenchymal tissue induced by implantation of demineralized bone powder (DBP) preincubated in phosphate buffered saline (PBS)  $\pm$  fibronectin in rat thoracic subcutaneous tissue. Magnesium content of the ash sample was analyzed by the method of Willis using atomic absorption spectrophotometry. Data are presented as an average of two samples for day 1 and the mean  $\pm$  S.D. of 3 samples for all other harvests.

# MAGNESIUM CONTENT

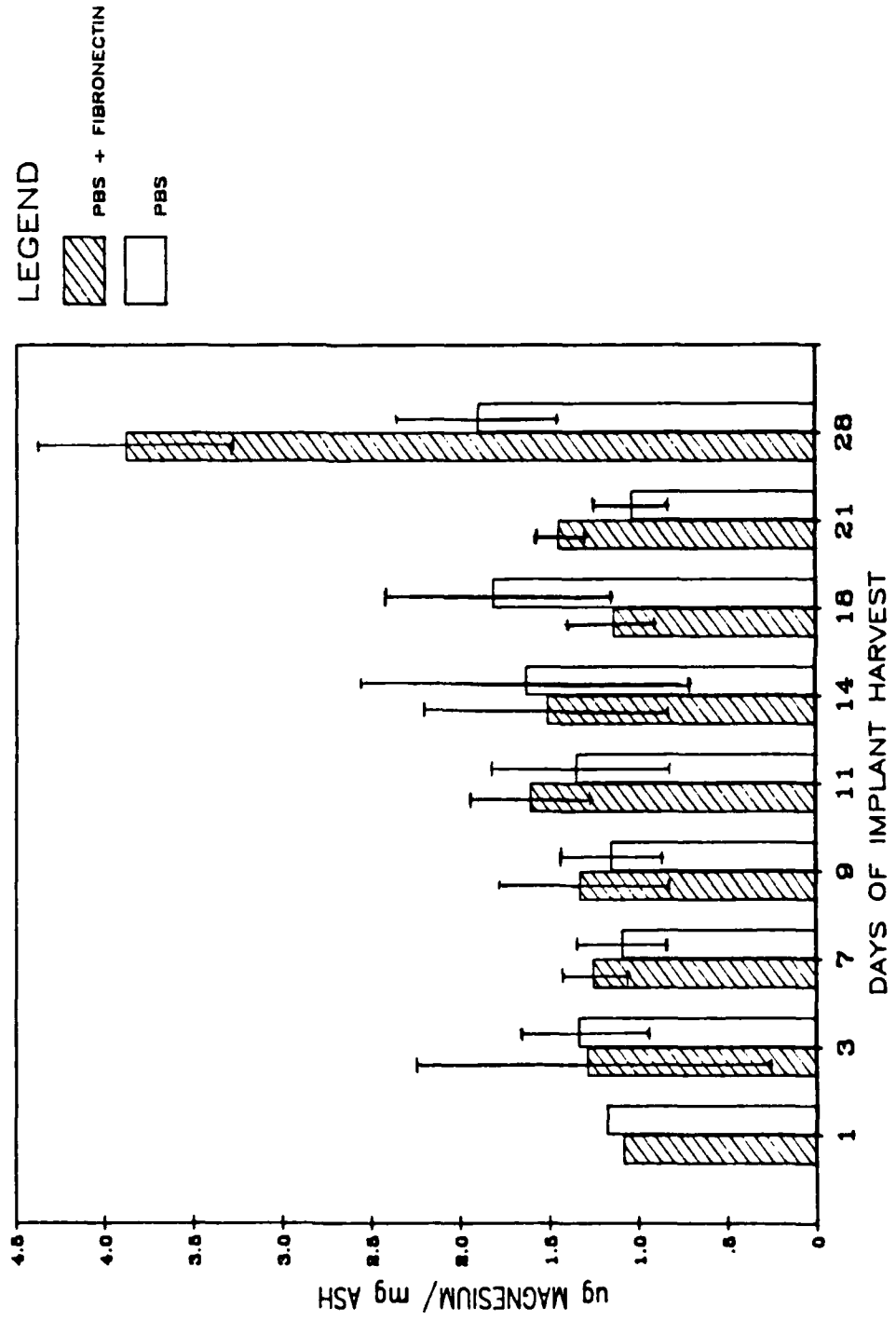


Figure 5. CHANGE IN THE CALCIUM CONCENTRATION OF THE ASH OF MESENCHYMAL TISSUE INDUCED BY IMPLANTATION OF DEMINERALIZED BONE POWDER (DBP)  $\pm$  FIBRONECTIN IN THE SUBCUTANEOUS FASCIA OF RAT THORAX.

The ash sample obtained from mesenchymal tissue induced by implantation of demineralized bone powder (DBP) preincubated in phosphate buffered saline (PBS)  $\pm$  fibronectin in subcutaneous tissues of rat thorax was analyzed for calcium content by the method of Willis using atomic absorption spectrophotometry. Data are presented as an average of two samples from day 1 and the mean  $\pm$  S.D. of 3 samples for all other harvests.

# CALCIUM CONTENT

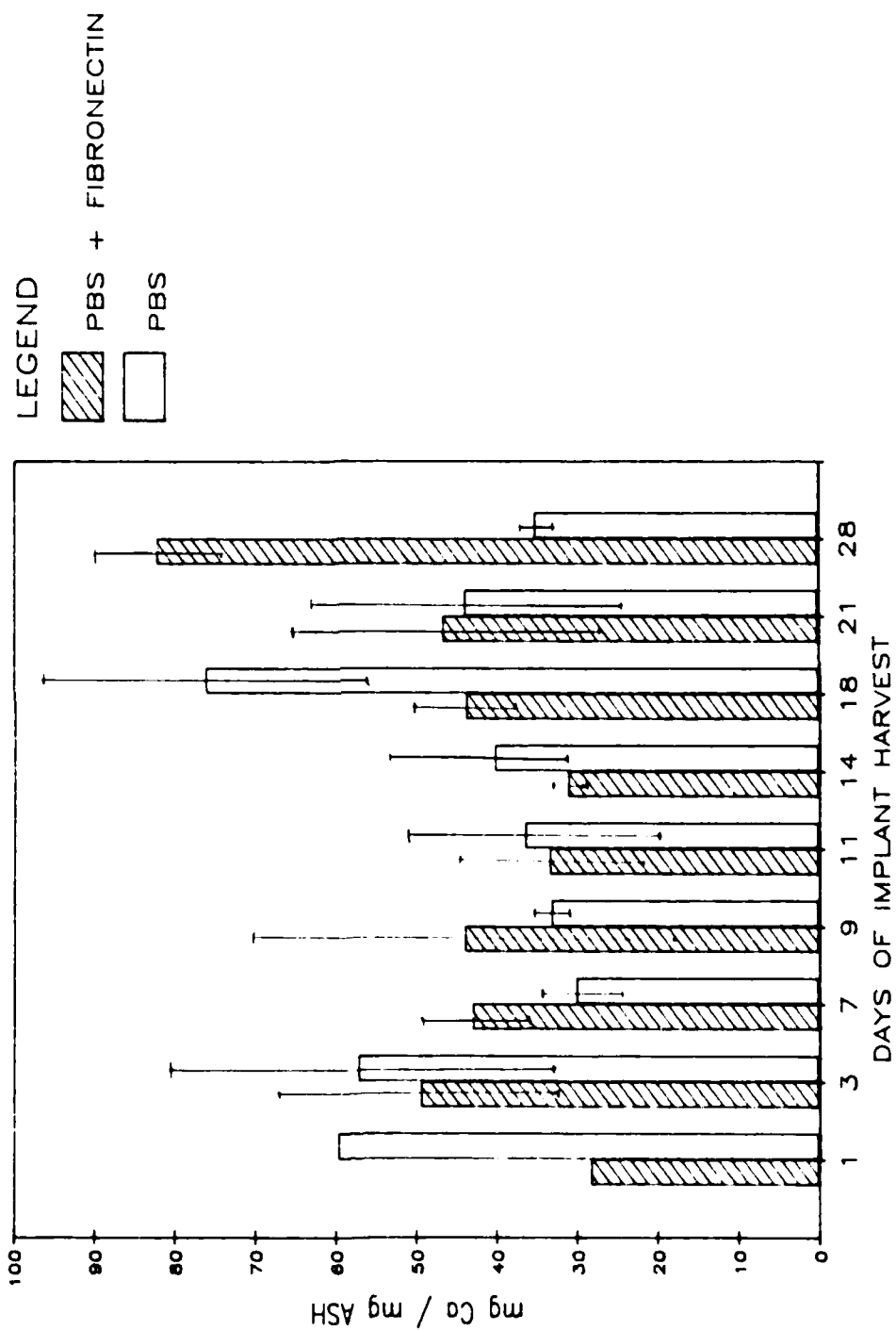


Figure 6. CHANGE IN THE PHOSPHATE CONCENTRATION OF THE ASH OF MESENCHYMAL TISSUE INDUCED BY IMPLANTATION OF DEMINERALIZED BONE POWDER (DBP)  $\pm$  FIBRONECTIN IN THE SUBCUTANEOUS FASCIA OF THE RAT THORAX.

The ash sample obtained from mesenchymal tissue induced by implantation of demineralized bone powder (DBP) preincubated in phosphate buffered saline (PBS)  $\pm$  fibronectin in subcutaneous tissues of the rat thorax harvested at 1, 3, 7, 9, 11, 14, 18, 21 and 28 days post implantation. The ash sample was analyzed for phosphate content colorimetrically by the method of Fiske and Subbarow. The data is expressed as the average of 2 samples from day 1 and the mean  $\pm$  S.D. of 3 samples for all other harvests.

# PHOSPHATE CONTENT

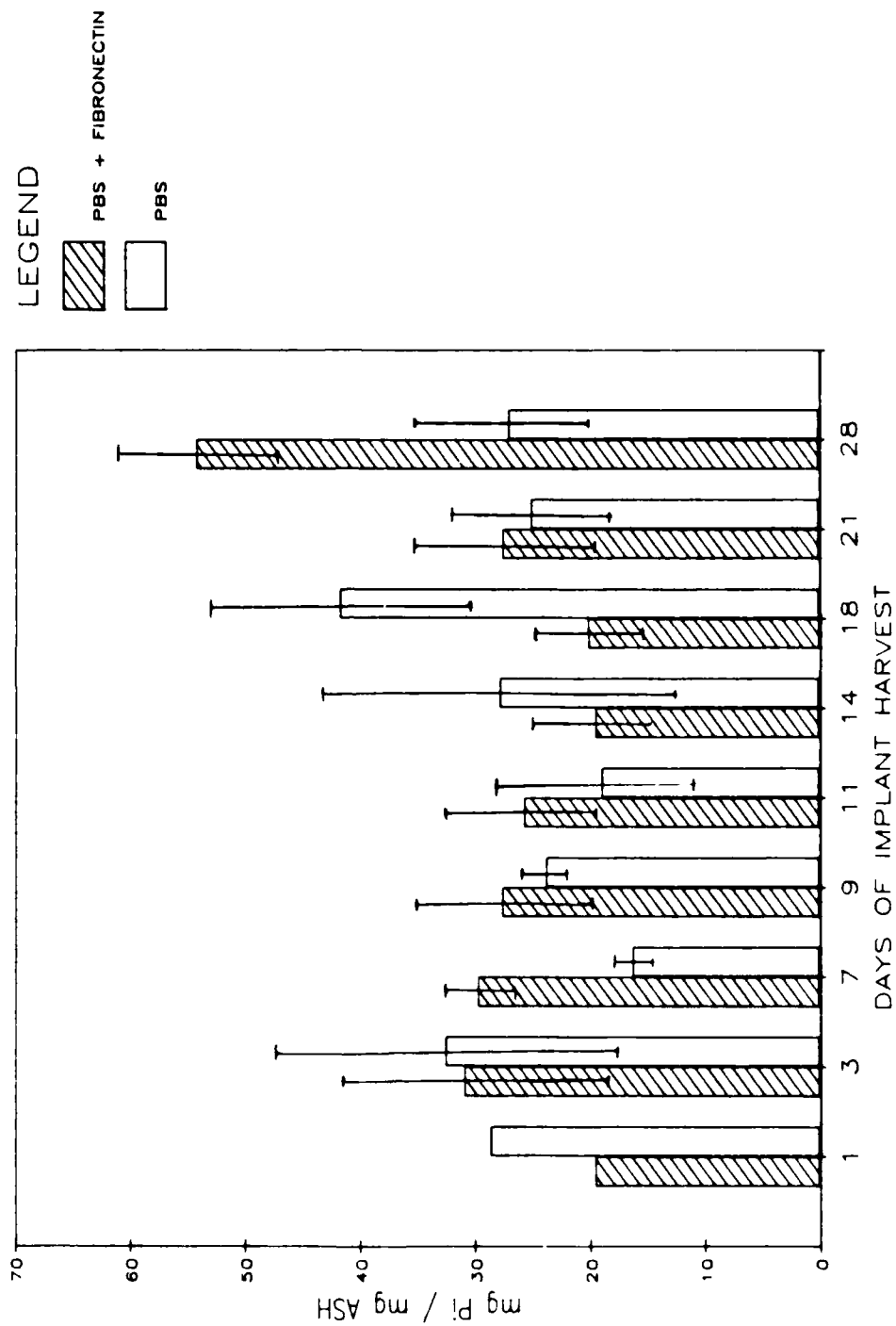
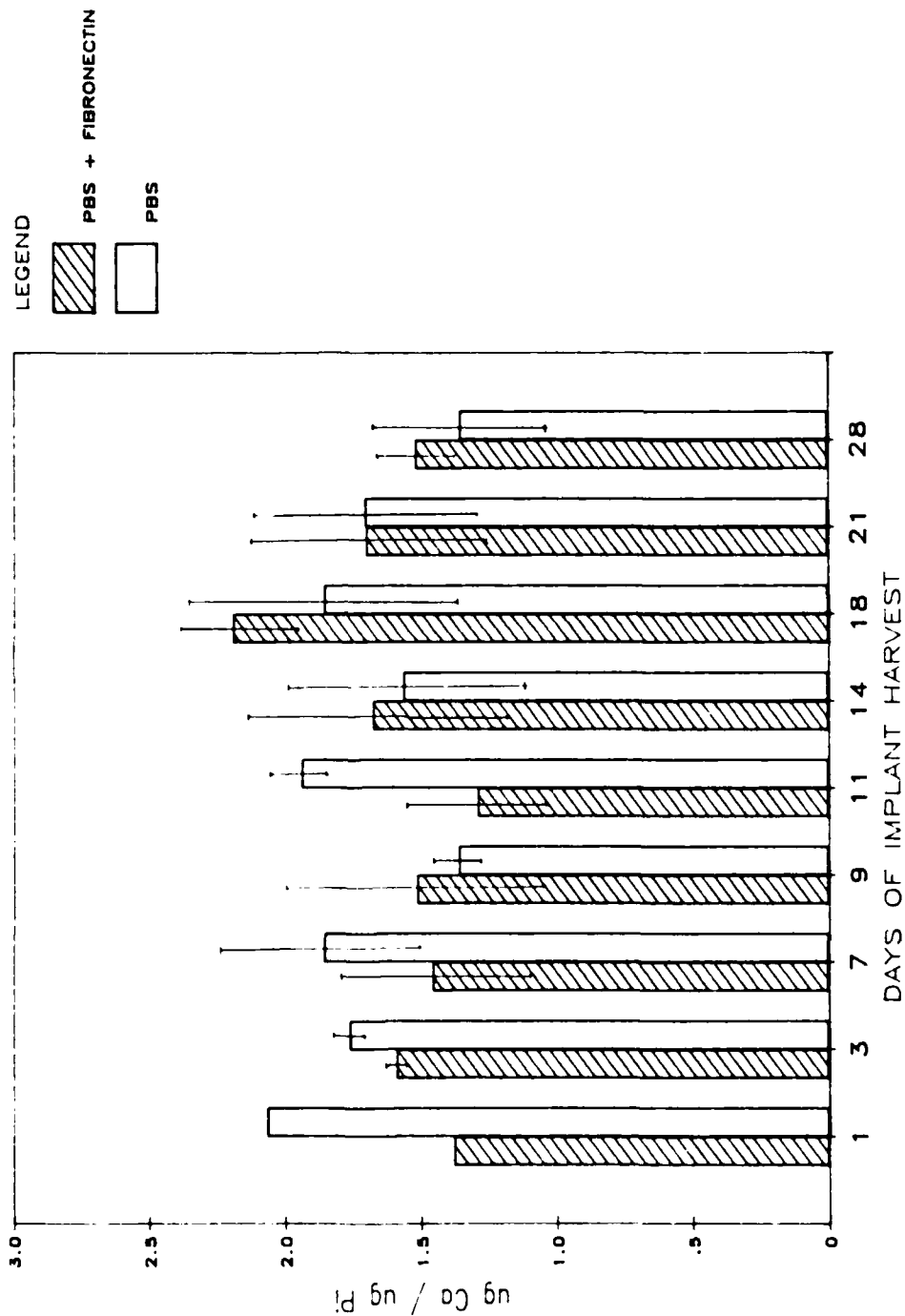


Figure 7. CHANGE IN THE CALCIUM/PHOSPHATE RATIO OF THE  
ASH OF MESENCHYMAL TISSUE INDUCED BY IMPLANTATION  
OF DEMINERALIZED BONE POWDER (DBP)  $\pm$  FIBRONECTIN  
IN SUBCUTANEOUS FASCIA OF RAT THORAX.

The ash sample was obtained from mesenchymal tissue induced by implantation of demineralized bone powder (DBP) preincubated in phosphate buffered saline (PBS)  $\pm$  fibronectin in subcutaneous tissues of rat thorax. The calcium/phosphate ratio is presented as an average of samples for day 1 and the mean ratio  $\pm$  S.D. of 3 samples for all other harvests.

# CALCIUM / PHOSPHATE RATIO



## V. DISCUSSION

The tissue response during wound healing is variable and includes a complex interaction consisting of many factors. In the tissue response following implantation of DBP, interactions occur between the quality and quantity of the implant material and the complex response system of the host. Our results suggest that subtle changes in the preparation of the DBP can markedly influence the mesenchymal tissue response. DBP incubated in PBS, whether or not fibronectin was present, failed to support endochondral ossification. Therefore, we examined the role that host age and the process that DBP 'wetting' had on the inductive process. Our observations are discussed below.

It has been determined that both the age of the donor and the age of the recipient affect the amount of cartilage and bone formed in response to the implantation of DBP (Urist, 1968, Irving, et al., 1981). Similar findings were published by Syftestad and Urist (1983) when 13 month old rats developed only 24% of the total cartilage that 3 month old rats produced from the implantation of the same bone matrix gelatin. In the current investigation, the recipient rats were about two months old when the DBP in PBS either with or without fibronectin was implanted into heterotopic thoracic sites, resulting in the tissues healing around the implants with a fibrous encapsulation. The tissue response of three week old rats to the DBP particles incubated in PBS

alone was identical to the response of the two month old rats; that is, fibrous encapsulation of the particles. Thus, the age of the two month old rat was not a factor in the inability of the tissues to respond to the osteoinductive features of the DBP particles.

Another factor which has been shown to affect the tissue response to the DBP is the geometry of the particles. The physical dimensions of the matrix particles have an important influence on the quantitative response of new bone formation (Reddi and Huggins, 1973). The optimal size for bone induction is in the range of 74-450um. These observations are consistent with known requirements for the dimensions of substratum in anchorage-dependent cell proliferation (Stoker, et al., 1968; Folkman and Moscona, 1978; Gospodarowicz, et al., 1980). It could be postulated from this information, that the relationship of the DBP particle to adjacent particles may influence the inductive stimuli. Thus, it is possible that the DBP particles need to be in relatively close proximity to each other. Addition of a wetting agent to the DBP particles may result in a less condensed mass of particles being implanted leading to a reduced osteoinductive influence. The implantation of the PBS dispersed particles could be one possible explanation for the lack of bone formation while the DBP implanted in a dry form resulted in cartilage and bone formation. The effect of wetting the DBP with PBS immediately prior to implantation versus the incubation for a 24 hour period has

not been addressed in this experimental model and may not result in the same response or may vary in the magnitude of the tissue response.

The physical preparation of the demineralized bone powder could have influenced the osteoinductive properties of the bone powder. In the preparation of the demineralized bone, the most important requirement for the matrix is that all acid-soluble components be removed (Glowacki, Altobelli and Mulliken, 1981). Mineral containing powders are not osteoinductive, but rather induce resorption by mono and multinucleated cells (Glowacki, Altobelli and Mulliken, 1981; Holtrop, Cox, and Glowacki, 1982). The protocol for the demineralization process (Kaban and Glowacki, 1981) requires copious rinses with deionized water after demineralization to remove all the mineral containing elements. Inadequate rinsing of the DBP could have afforded a possible explanation of the results leading to fibrosis in the first experiment when the DBP was placed using a wetting agent. However, in the second experiment when the DBP was implanted dry, the adjacent tissues produced bone via the endochondral pathway. The bone particles used throughout this investigation were all prepared and demineralized at one time. Therefore, adequate preparation of the demineralized bone powder appears to have been accomplished.

Sterilization procedures are known to affect the osteoinductive properties of the implant (Urist, et al., 1975; Urist, 1965; Towle, et al., 1986). In this experiment,

ethylene oxide was used to sterilize the DBP prior to implantation. Similar sterilization procedures were followed in all experimental models, therefore, it does not appear that the sterilization process affected the ability of the DBP particles to be osteoinductive since the DBP implanted in a dry form produced bone formation. However, this method of sterilization may have affected the magnitude of the bone formation as reported by Towle, et al. (1986), but that was not a parameter studied in this investigation.

The biochemical assays which were selected for this investigation assess whether and when mineral was being deposited in the implant site. Consequently, the biochemical data are difficult to correlate with the histological characterization, since the selected tests assess the formation of bone rather than fibrosis. However, there are some particular points of interest when correlating the biochemical and histologic characteristics.

Increases in alkaline phosphatase activity has been shown to precede incorporation of calcium uptake into the tissue (Reddi, et al., 1972). Thus, peaks in alkaline phosphatase are used as an indicator of mineralization. Investigations (Reddi and Anderson, 1976; Reddi and Huggins, 1972) using DBP implanted into subcutaneous sites of Long Evans rats, similar to this investigation, have shown two peaks in a twenty-eight day time frame. The first peak occurred at about Day 8 when cartilage was formed and a second higher peak occurred at about Day 10-11 and stayed

high until about Day 14. The increased alkaline phosphatase activity correlated with increased incorporation of  $^{45}\text{Ca}$  uptake in the tissues surrounding the DBP particles. In contrast, the current investigation showed that when the DBP particles were implanted after being incubated in PBS alone or PBS containing fibronectin, the peak in alkaline phosphatase occurred at Day 14. Although the data does not support a statistically significant increase in the calcium at or near Day 14, the histologic presentation of hyalinized tissue or early woven bone is observed in histologic sections from Day 14 (see plate 2, figure 2).

The correlation of the increased alkaline phosphatase and the histologic presentation of a hyalinized or early woven bone could represent a delayed response of the tissues to mineralize. If mineralization was trying to occur, it may be representative of a dystrophic calcification. In any event, if attempted mineralization of the tissues was made, it was aborted and the final result was a fibrotic encapsulation of the DBP particles.

The wetting (and possibly the addition of the incubation time) of the DBP particles with PBS appears to be the factor influencing the osteoinductive influences of the DBP particles. A possible explanation for this result could be that the saline "leached" out the osteoinductive factors present in the DBP. In other investigations (Syftestad, et al., 1984), saline has been used to remove osteoinductive proteins from DBP, but in that experiment the bone powder

had previously been extracted with guanidine which is a dissociative solvent. The DBP in this investigation was incubated in PBS for 24 hours prior to implantation and this procedure could have caused an extraction of one or more of the inductive factors.

A second more plausible explanation of the inhibitory effects that PBS may have on the osteoinductive properties of the DBP relates to the proximity of the DBP particles when they are implanted. The addition of PBS, or any 'wetting' agent may affect the ability of the particles to be condensed in the same tight mass as the dry powder particles. The relative position of one particle to another may be the critical factor. The compacted particles may be necessary to concentrate the osteoinductive factor to a critical level which is necessary for bone induction. Thus, the addition of PBS may indirectly cause a 'dilution' of the inductive factor, since the distance from the responding cell population may be greater than the diffusion of the of the osteoinductive factor.

The addition of exogenous fibronectin in PBS did not alter the resulting fibrosis, nor did it appear to enhance the fibrosis. The control (PBS only) and the experimental (PBS containing fibronectin) models progressed similarly. The decision to use fibronectin in PBS rather than a powdered, lyophilized form of fibronectin was made since it is known that the lyophilization of the fibronectin destroys its biologic activity (personal communication, R. Klebe,

Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio). In addition, possible implications relating to clinical applications should be addressed. It is a common clinical practice to add a 'wetting' agent to bone powders prior to implantation to allow for easier handling of the material. In the situation where DBP particles are used as the implant material, the wetting of the material may affect the osteoinductive properties and thus, the clinical results may be affected. The variation in the technique of placing DBP 'wetted' in contrast to a 'dry' powder could make a difference in the clinical results of healing by fibrosis versus healing by osteoinduction. This would be of particular interest to the clinician if it is could be determined by future experimentation that the wetting of the DBP just prior to implantation has a similar effect to that of incubating it for 24 hours in saline prior to implantation.

## VI. CONCLUSIONS

A. Incubation of demineralized bone powder in a wetting agent for a period of 24 hours inhibits the osteoinductive properties of the hetertopically implanted material, resulting in fibrosis.

B. The age of the recipient rat did not appear to be a factor in tissue response to 'wetted' DBP implants. The inhibitory effect was demonstrated in 3 week old rats weighing 100-120 grams as well as in 2 month old rats weighing 240 grams at time of implantation.

C. The demineralized bone powder as prepared in this investigation is osteoinductive when implanted in a dry form in a hetertopic site.

D. The results of this investigation were unable to determine the effect of the addition of exogenous fibronectin on the heterotopically placed bone particles, other than as a feature of the fibronectin in PBS acting as a wetting agent. In this form, fibronectin did not alter the mesenchymal tissue response to the DBP in any different manner than the addition of PBS only.

APPENDIX

## PROTEIN CONTENT

	mg Protein per implant	MEAN	SD	+SD	-SD
DAY 1-Y-A	3.6755				
DAY 1-Y-B	4.7445	4.2100	.7539	4.9659	3.4541
DAY 1-B-A	3.0130				
DAY 1-B-B	4.6260	3.9195	1.2820	5.2015	2.6375
DAY 3-Y-A	3.1045				
DAY 3-Y-B	3.3140				
DAY 3-Y-C	4.8025	3.7403	.9258	4.6661	2.8145
DAY 3-B-A	1.1010				
DAY 3-B-B	1.6255				
DAY 3-B-C	1.5240	1.4168	.2790	1.6948	1.1388
DAY 7-Y-A	2.5050				
DAY 7-Y-B	2.6145				
DAY 7-Y-C	2.6140	2.5778	.0631	2.6409	2.5147
DAY 7-B-A	2.3725				
DAY 7-B-B	2.2840				
DAY 7-B-C	1.8090	2.1552	.3030	2.4582	1.8522
DAY 9-Y-A	2.1865				
DAY 9-Y-B	2.3055				
DAY 9-Y-C	2.8090	2.4337	.3304	2.7641	2.1033
DAY 9-B-A	1.6330				
DAY 9-B-B	3.3960				
DAY 9-B-C	1.8290	2.2860	.9663	3.2523	1.3197
DAY 11-Y-A	3.1585				
DAY 11-Y-B	3.2380				
DAY 11-Y-C	2.3730	2.9232	.4781	3.4013	2.4451
DAY 11-B-A	3.8035				
DAY 11-B-B	4.0085				
DAY 11-B-C	3.6005	3.8042	.2040	4.0082	3.6002
DAY 14-Y-A	1.9515				
DAY 14-Y-B	1.7750				
DAY 14-Y-C	1.7540	1.8202	.1156	1.9358	1.7046
DAY 14-B-A	2.1970				
DAY 14-B-B	2.4565				
DAY 14-B-C	1.5265	2.0600	.4799	2.5399	1.5801
DAY 18-Y-A	1.9225				
DAY 18-Y-B	1.9965				
DAY 18-Y-C	1.5025	1.8072	.2841	2.0913	1.5231
DAY 18-B-A	1.3925				
DAY 18-B-B	2.0110				
DAY 18-B-C	1.6745	1.6927	.3097	2.0024	1.3830
DAY 21-Y-A	1.0735				
DAY 21-Y-B	1.8930				
DAY 21-Y-C	1.6000	1.5222	.4153	1.9375	1.1069
DAY 21-B-A	1.5510				
DAY 21-B-B	2.8315				
DAY 21-B-C	2.6850	2.3558	.7008	3.0566	1.6550
DAY 28-Y-A	.6075				
DAY 28-Y-B	.7240				
DAY 28-Y-C	.6640	.6652	.0583	.7235	.6069
DAY 28-B-A	1.3150				
DAY 28-B-B	1.6545				
DAY 28-B-C	1.6595	1.5437	.1980	1.7417	1.3457
GROUP	SUM OF SQUARES	DF	MEAN-SQUARE	F-RATIO	P
DAY	37.8332	1	37.8332	.1410	.7100
	31,608.1000	8	3,951.0075	14.6740	.0001
COMPARISONS OF UNEQUAL SAMPLE SIZE		LSI			
	2X2	1.0897			
	2X3	.9949			
	3X3	.8897			







## CALCIUM CONTENT

	ug Ca/mg Ash	MEAN	SD	+SD	-SD
DAY 1-Y-A	39.8678				
DAY 1-Y-B	16.9048	28.3863	16.2470	44.6283	12.1443
DAY 1-B-A	47.5610				
DAY 1-B-B	72.0120	59.7865	17.2640	77.0757	42.4975
DAY 3-Y-A	36.8056				
DAY 3-Y-B	68.7500				
DAY 3-Y-C	43.0350	49.5312	16.9310	66.4622	32.6002
DAY 3-B-A	44.1550				
DAY 3-B-B	84.1463				
DAY 3-B-C	43.4783	57.2601	23.2870	80.5471	33.9731
DAY 7-Y-A	49.2925				
DAY 7-Y-B	35.9375				
DAY 7-Y-C	43.9759	43.0686	6.7210	49.7896	36.3476
DAY 7-B-A	31.6667				
DAY 7-B-B	33.3333				
DAY 7-B-C	25.6522	30.2174	4.0410	34.2584	26.1764
DAY 9-Y-A	73.7569				
DAY 9-Y-B	30.7339				
DAY 9-Y-C	27.7344	44.0751	25.7530	69.8281	18.3221
DAY 9-B-A	33.3333				
DAY 9-B-B	30.7407				
DAY 9-B-C	35.7759	33.2833	2.5246	35.8033	30.7633
DAY 11-Y-A	45.9770				
DAY 11-Y-B	24.0458				
DAY 11-Y-C	30.5756	33.5261	11.2650	44.7891	22.2631
DAY 11-B-A	32.8947				
DAY 11-B-B	24.2092				
DAY 11-B-C	52.0558	36.5876	13.9460	50.5836	22.5916
DAY 14-Y-A	33.3333				
DAY 14-Y-B	29.0076				
DAY 14-Y-C	31.3492	31.2301	2.1620	33.3921	29.0681
DAY 14-B-A	51.3158				
DAY 14-B-B	43.8931				
DAY 14-B-C	25.8624	40.3638	13.6820	53.4458	27.2818
DAY 18-Y-A	36.9792				
DAY 18-Y-B	48.4472				
DAY 18-Y-C	46.3918	43.9394	6.1150	50.0544	37.8144
DAY 18-B-A	90.9091				
DAY 18-B-B	83.7838				
DAY 18-B-C	53.8835	76.1421	19.6470	95.8391	56.4351
DAY 21-Y-A	68.2540				
DAY 21-Y-B	35.1852				
DAY 21-Y-C	36.9048	46.7013	18.6160	65.3173	28.0853
DAY 21-B-A	25.0000				
DAY 21-B-B	43.8462				
DAY 21-B-C	63.3929	44.0797	19.1460	63.2257	24.9337
DAY 28-Y-A	83.3333				
DAY 28-Y-B	73.9130				
DAY 28-Y-C	89.5633	62.2766	26.9440	89.2206	35.3326
DAY 28-B-A	35.5556				
DAY 28-B-B	36.9565				
DAY 28-B-C	33.6364	35.3828	6.7470	42.1298	28.6358

GROUP	SUM OF SQUARES	DF	MEAN SQUARE	F
DAY	16.8920	1	16.8920	1.00
DAY	4,689,7070	8	586,213.375	34.80

COMPARISONS OF UNEQUAL SAMPLE SIZE	1-2	1-3	1-4	2-3	2-4	3-4
2X3						
3X3						

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MESENCHYMAL TISSUE RESPONSE TO HETEROTOPICALLY PLACED  
DEMINEALIZED BONE. (U) AIR FORCE INST OF TECH  
WRIGHT-PATTERSON AFB OH F M FEGLEY 1987

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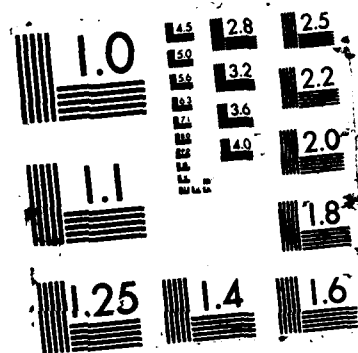
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## PHOSPHATE CONTENT

	ug P1/ug Ash	RE/M	50	100	-50
DAY 1-Y-A	23.2339	19.6593	5.0790	24.7183	14.6903
DAY 1-Y-B	16.0840				
DAY 1-B-A	26.1841	26.7993	.0130	29.5723	27.9463
DAY 1-B-B	24.3345				
DAY 3-Y-A	23.4569				
DAY 3-Y-B	42.9029	31.0162	10.4640	41.4852	20.5582
DAY 3-Y-C	26.8291				
DAY 3-B-A	25.0117				
DAY 3-B-B	46.1415	32.6619	14.2840	46.9459	14.3779
DAY 3-B-C	23.0226				
DAY 7-Y-A	27.3934				
DAY 7-Y-B	22.0940	29.8264	2.3590	32.1854	27.4674
DAY 7-Y-C	26.6897				
DAY 7-B-A	14.6475				
DAY 7-B-B	17.1189	16.4021	1.6090	18.0121	14.8021
DAY 7-B-C	17.3200				
DAY 9-Y-A	35.8203				
DAY 9-Y-B	21.7408	27.6868	7.3090	34.9958	20.3770
DAY 9-Y-C	29.4633				
DAY 9-B-A	25.6792				
DAY 9-B-B	21.7281	23.6409	2.0020	25.6429	21.6699
DAY 9-B-C	24.2655				
DAY 11-Y-A	32.6943				
DAY 11-Y-B	32.9748	29.7876	6.2020	31.9896	19.5856
DAY 11-Y-C	20.6937				
DAY 11-B-A	16.7140				
DAY 11-B-B	12.6464	19.0633	8.3750	27.3383	10.7803
DAY 11-B-C	28.4094				
DAY 14-Y-A	18.3908				
DAY 14-Y-B	25.3321	19.3910	9.2440	24.8350	14.3470
DAY 14-Y-C	19.0700				
DAY 14-B-A	47.2246				
DAY 14-B-B	21.7076	27.8890	15.2160	43.1040	12.6720
DAY 14-B-C	16.7318				
DAY 15-Y-A	15.4323				
DAY 15-Y-B	22.0849	20.2441	4.2070	24.4511	16.0371
DAY 15-Y-C	32.2155				
DAY 15-B-A	34.6231				
DAY 15-B-B	34.4941	41.7912	11.1110	52.9022	30.6802
DAY 15-B-C	33.6873				
DAY 21-Y-A	34.5603				
DAY 21-Y-B	29.2642	27.6267	7.0800	35.5147	19.7107
DAY 21-Y-C	19.0476				
DAY 21-B-A	20.2649				
DAY 21-B-B	22.1641	25.1732	6.9050	32.1072	18.2462
DAY 21-B-C	33.0688				
DAY 26-Y-A	60.9056				
DAY 26-Y-B	47.6652	94.2972	6.6200	60.9172	47.6772
DAY 26-Y-C	54.3208				
DAY 26-B-A	35.9556				
DAY 26-B-B	23.1894	27.1231	7.3040	34.4321	19.0141
DAY 26-B-C	22.6235				
GROUP	SUM OF SQUARES	DF	MEAN	SOURCE	P
DAY	26.5970	1			
	1,657.2150	8			
COMPARISONS OF UNIFORM SAMPLE SIZE	150				
2N3	14.8900				.5270
3N3	13.2400				.0090

## CALCIUM/PHOSPHATE RATIO

	ug Ca/ug P	mean	SD	-SD	+SD
DAY 1-Y-A	1.7159				
DAY 1-Y-B	1.0510	1.3435	.4740	.9095	1.8575
DAY 1-B-A	1.4875				
DAY 1-B-B	2.4549	2.0712	.5370	1.5342	2.6082
DAY 3-Y-A	1.5491				
DAY 3-Y-B	1.6002	1.8952	.0210	1.8742	1.9162
DAY 3-Y-C	1.6142				
DAY 3-B-A	1.7654				
DAY 3-B-B	1.7123	1.7674	.0550	1.7124	1.8224
DAY 3-B-C	1.8243				
DAY 7-Y-A	1.7994				
DAY 7-Y-B	1.1196	1.4618	.3400	1.1218	1.8018
DAY 7-Y-C	1.5653				
DAY 7-B-A	2.1725				
DAY 7-B-B	1.5472	1.8617	.3430	1.4987	2.2247
DAY 7-B-C	1.4642				
DAY 9-Y-A	2.0074				
DAY 9-Y-B	1.4133	1.8199	.4940	1.0259	2.6139
DAY 9-Y-C	2.0692				
DAY 9-B-A	1.2081				
DAY 9-B-B	1.4148	1.3957	.0840	1.3097	1.4817
DAY 9-B-C	1.4743				
DAY 11-Y-A	1.4043				
DAY 11-Y-B	1.0030	1.2953	.2990	1.0363	1.5543
DAY 11-Y-C	1.4744				
DAY 11-B-A	2.0414				
DAY 11-B-B	1.9287	1.9442	.1040	1.8382	2.0502
DAY 11-B-C	1.8324				
DAY 14-Y-A	1.8125				
DAY 14-Y-B	1.1431	1.6802	.4780	1.2022	2.1582
DAY 14-Y-C	2.0830				
DAY 14-B-A	1.1347				
DAY 14-B-B	2.0220	1.8479	.4450	1.1229	2.0129
DAY 14-B-C	1.5489				
DAY 18-Y-A	2.3942				
DAY 18-Y-B	2.1937	2.1961	.2000	1.9961	2.3961
DAY 18-Y-C	1.9983				
DAY 18-B-A	2.4397				
DAY 18-B-B	1.3384	1.8098	.5030	1.3568	2.3628
DAY 18-B-C	1.6009				
DAY 21-Y-A	1.9745				
DAY 21-Y-B	1.2023	1.7048	.4360	1.2688	2.1408
DAY 21-Y-C	1.9375				
DAY 21-B-A	1.2335				
DAY 21-B-B	1.9768	1.7090	.4170	1.2920	2.1260
DAY 21-B-C	1.9170				
DAY 28-Y-A	1.3682				
DAY 28-Y-B	1.9507	1.9227	.1420	1.7807	1.6647
DAY 28-Y-C	1.6492				
DAY 28-B-A	1.8000				
DAY 28-B-B	1.9518	1.3601	.3160	1.0441	1.6761
DAY 28-B-C	1.4867				
GROUP	SUM OF SQUARE	DF	MEAN SQUARE	F RATIO	P
DAY	.2160	1	.2160	1.7940	.1890
	1.3948	8	.1738	1.4640	.2100

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